ANTIBACTERIAL ACTIVITY OF FRESHWATER GREEN MICROALGAE ISOLATED FROM WATER BODIES NEAR ABANDONED MINE SITES IN ONTARIO, CANADA

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ABSTRACT

Progress of modern medicine relies on the discovery of new antibiotics. The increasing threat of antibiotic resistant bacteria, leading to an increase in morbidity and mortality of patients previously considered low risk, has highlighted the serious need for the expansion of antibiotic research and development. Historically, natural products have been the most successful source of antibiotics as they have complex and unique chemical structures and modes of action. Since most available antibiotics are originally a result of the secondary metabolism of bacteria and fungi, microorganisms from diverse environments capable of producing secondary metabolites have been and currently are being investigated for antibacterial production.

Green microalgae are eukaryotic microorganisms that can be found in a very wide range of habitats, including extreme environments. These microorganisms are known to produce a series of commercially valuable compounds as a result of their secondary metabolism. The central aim of this thesis was to determine the potential of green microalgae as antibiotic producers collected (bioprospected) from water bodies near abandoned mine sites in Ontario, Canada. These water bodies exhibited a variety of chemical profiles, including high metal concentrations and low pH.

Forty species of green microalgae were subsequently isolated and their extracts tested against various bacteria. The findings showed that 37.5% of these microalgae produced antibacterial compounds that seem to specifically inhibit the growth of Gram-positive bacteria, in particular the opportunistic pathogen *Staphylococcus aureus*. This was a higher success rate than any previous study on green microalgae. In addition, the evaluation of crude extracts of *Chlamydomonas* sp., the most common isolated species, demonstrated variation in antibacterial

activity during cell growth. The highest antibacterial activity from this species was found in the exponential phase.

Furthermore, green microalgal extracts exhibiting antibacterial activity also decreased the cell viability of malignant cells, particularly the rapidly dividing human ovarian carcinoma A2780 cells. However, the extracts did not decrease the cell viability of non-malignant cells. Taken together, the results of this thesis reveal that freshwater green microalgae from water bodies near abandoned mine sites are potential sources of antibacterial compounds against Gram-positive bacteria and should be further investigated against rapidly dividing malignant cells.

Keywords: abandoned mine sites, antibiotic, antibacterial activity, freshwater, green microalgae, viability, malignant cells.

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LIST OF ABBREVIATIONS

2D	two dimensional
AMD	acid mine drainage
AMIS	abandoned mines information system
ATCC	American tissue culture collection
BBM	Bold's basal medium
C	chromatography
Cr	*Chlamydomonas reihardtii* CPCC11
DART-MS	direct analysis in real time mass spectrometry
DI-LC/MS/MS	direct injection liquid chromatography tandem mass spectrometry
DMSO	dimethyl sulfoxide
EIMS	electron impact mass spectrometry
ESIMS	electrospray ionization mass spectrometry
GC	gas chromatography
HREIMS	high resolution electron impact mass spectrometry
HRMS	high-resolution mass spectrometry
IC	ion chromatography
IC_{50}	half maximal inhibitory concentration
IR	infrared
LC-MS	liquid chromatography mass spectrometry
MALDI-TOF/MS	matrix assisted laser desorption ionization-time of flight tandem mass spectrometry

MH	Muller Hinton
MIC	minimum inhibitory concentration
MPLC	medium pressure liquid chromatography
MRSA	methicillin resistant *Staphylococcus aureus*
MS	mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	nuclear magnetic resonance
NPVLC	normal phase vacuum liquid chromatography
PBS	phosphate buffered saline
R-P-HPLC	reversed phase high performance liquid chromatography
Sd	*Scenedesmus dimorphus UTEX* 1237
SPE	solid phase extraction
TLC	thin layer chromatography
UTEX	culture collection of algae at the University of Texas at Austin
UV	ultraviolet visible

CHAPTER 1: INTRODUCTION

1.1 The "Golden Era" and the lack of new classes of antibiotics

The discovery of antibiotics represents undoubtedly the major scientific achievement against deadly bacterial infections. Antibiotics have impacted medicine not only by curing infections and saving millions of lives, but also by preventing infections in chronic diseases, immunosuppression and extensive surgeries (Ventola 2015). The impact of antibiotic therapy on health care is reflected in changes in life expectancy (Wright 2014). For example, in Canada, life expectancy in the early 1920s for men was 58.8 years of age, whilst recently it is 78.3, and for women it was 60.6 and now 83 (Statistics Canada, 2012).

The time between 1940 and 1960 is regarded as the "Golden Era" of antibiotics, as most available classes of antibiotic were discovered based on natural product screening (Fernandes 2006). Soil actinomycetes and fungi were screened for antibacterial activity against test bacteria by measuring the zones of growth inhibition on agar plates (Lewis 2013). During this time, many new antibacterial drugs were discovered and became commercially available (Figure 1.1), generating a feeling of control over bacterial infections (Fernandes 2006). However, in the early 1960s, bacterial resistance to antibiotics started to emerge and spread into hospital settings (Fernandes 2006).

The search for new antibacterial compounds after the 1960's based on natural product screening only resulted in rediscovery of known compounds (Silver 2011). As bacterial resistance to available drugs increased, other solutions had to be pursued (Lewis 2013). This included research on antibiotic analogs (Fernandes 2006) based on chemical modifications of previous

discovered antibiotics (Clardy *et al.* 2006). However, over two decades with these potent analogs, bacteria have evolved and circumvented their antibacterial activity, and, therefore, new classes of antibiotics are again needed (Coates *et al.* 2011).

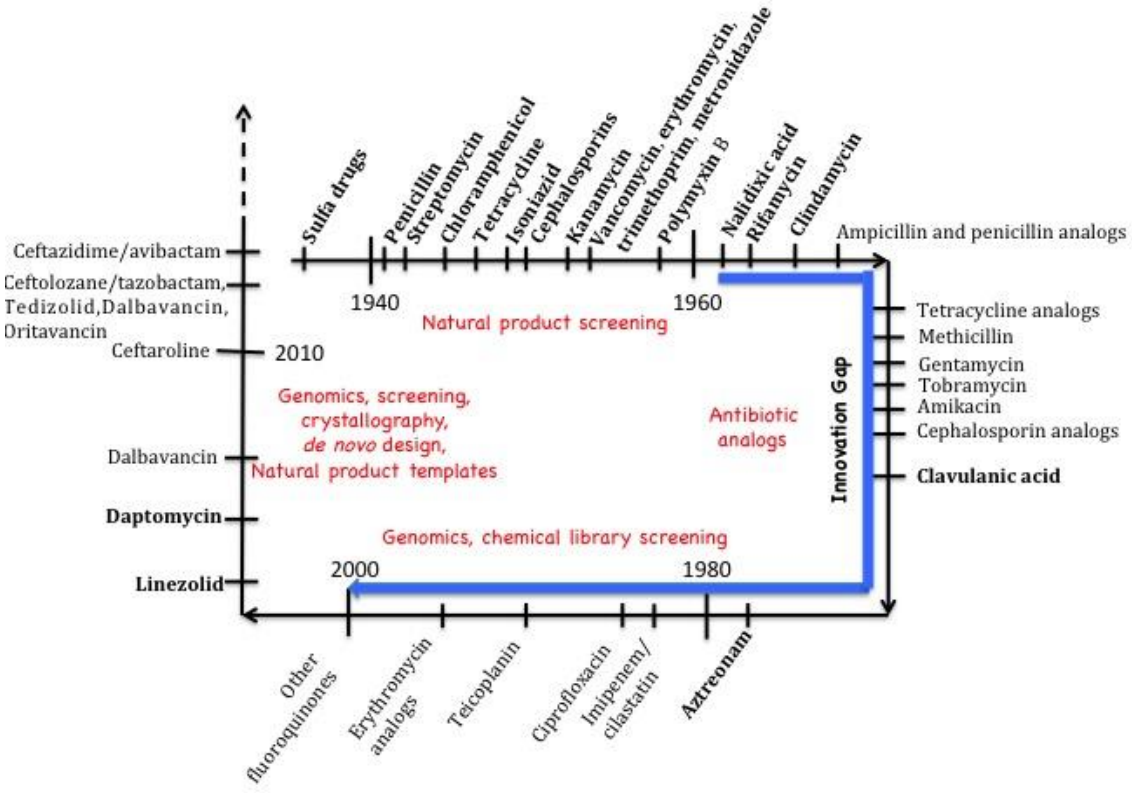

Figure 1.1- Timeline of most clinically used antibiotic investigation. Most major new classes of antibiotics were discovered and commercialized until 1961. Even though different technologies were applied no major classes of antibiotics were introduced from 1962 to 2000. Technologies used in antibiotic discovery are shown in red letters and new antibiotic classes in bold letters. The blue arrow shows the "innovation gap" (Adapted from Fernandes 2006, Fischbach & Walsh 2009, Fernandes & Martens 2017).

The four decades that followed the "Golden Era" have been referred to as the "innovation gap", as no major new classes of antibiotics were discovered (Fischbach & Walsh 2009, Figure 1.1). This was certainly not due to a lack of technology, but in part as a result of the decrease in the commercial attractiveness of the antibiotic market after the "easier" antibiotic classes had been discovered (Stanton 2013). With the goal of maximizing profitability, pharmaceutical companies redirected their resources to long-term use drugs that treat chronic diseases such as cancer, diabetes and cardiovascular diseases (Stanton 2013).

In the 1980s, the companies still working on natural product screening switched efforts to the synthetic chemical route of small-molecule library screening due to the low probability of success and diminished productivity (Clardy *et al.* 2006). The focus was to identify bacterial proteins as potential targets for antibacterial compounds and screen for inhibitors (Fernandes 2006). Even though promising enzyme inhibitors were obtained, issues regarding permeability, pharmacokinetics and toxicity have proven these inhibitors to be poor antibiotics (Silver 2011).

This diminished "pipeline" of antibiotics has led to only a few classes of antibiotics commercialized since the 1970s, such as oxazolidinones and lipopeptides (Fernandes 2006). Although both these antibiotic classes were initially commercialized in the early 2000s, their discoveries date back to the 1970s and 1980s, respectively (Silver 2011). In order to discover new antibacterial compounds, a variety of technologies have been employed in the last two decades, including genomics, bioinformatics, combinatorial biology, cloning, crystallography, and high-throughput methods (Silver 2011). Although promising leads have been discovered, the rate of success has been very low (Fernandes 2017).

These new technologies have not provided the hoped for "magic bullet" to generate much needed new antibiotics (Donadio *et al.* 2010), and the spread of multidrug resistant bacteria is continually increasing (Rokem 2007). According to the Centers for Disease Control and Prevention (CDC 2013), antibiotic resistant bacteria kill in United States at least 23,000 people each year as a direct result from the infections and contribute to two million illnesses. It is clear, therefore, that if the pressing need for new antibiotics is not met, a return to the pre-antibiotic like era is possible (Chopra 2013).

However, considering that over two thirds of available antibiotics are the result of natural products or their derivatives (Fischbach & Walsh 2009), it is reasonable to assume that organisms are still excellent potential sources of these compounds. Furthermore, advanced technologies, including high-throughput screening, cultivation of unknown microorganisms, metabolomics and genome mining, are now available to make this investigation easier than in the past (Kealey *et al.* 2017). Therefore, natural product screening should not be abandoned (Wright 2014).

Even though many research chemists do not like to work with natural compounds due to their complexity, it is unquestionable that natural compounds can have advantages over synthetic ones. These include an ability to penetrate bacterial cells (Sheridan 2006). Other advantages of natural products may also include (Lam 2007):

- unrivaled diversity promoting chemical complexity and biological activity;
- use as a template in combinatorial chemistry for antibiotic analogs production;
- possession of desirable pharmacokinetic properties.

As only a very small fraction of the world's biodiversity has been explored (Sheridan 2006), a small but growing number of studies have been focusing on the antibacterial potential of unexplored microorganisms, and they have shown that there is hope for antibiotic discovery. Ling *et al.* (2015) discovered teixobactin, a promising new class of antibiotic isolated from a previously unknown and uncultured bacterium from soil, which showed activity against methicillin-resistant *Staphylococcus aureus*. Maffioli *et al.* 2017 discovered a new broad-spectrum antibiotic that inhibits RNA polymerase from extracts of soil actinobacteria. However, the antibiotics that are in late clinical development are still not enough, and more new active compounds need to be found (Fernandes 2017).

In summary, there is a critical need for the continuous production of new antibiotics to combat the ability of pathogenic bacteria to evolve resistances. As new technologies are available to make the process easier, a potential solution is to go back to researching natural sources, particularly from previously unexplored microorganisms.

1.2 Microalgae – an overview

Algae are a heterogeneous group of thallophytes (plant-like organisms without differentiation into roots, stems and leaves). They conduct oxygen-evolving photosynthesis based upon chlorophyll *a* as their primary photosynthetic pigment, and have reproductive cells that lack a sterile covering of protecting cells (Lee 2008; Andersen 2013). These organisms are mostly autotrophic, generating complex organic molecules from inorganic sources (Bellinger & Sigee 2010) and are associated with the evolution of life on Earth, since they remove CO_2 and release O_2 (Demirbas & Demirbas 2010).

The classification of algae is not as straightforward as it is for other organisms, since they do not represent a formal taxonomic group of organisms. They, instead, are an assemblage of phyla or divisions based on a combination of cellular characteristics (Sheath & Wehr 2003), including photosynthetic pigments and reserve products (Sheath & Wehr 2015). According to Lee (2008), the algae phyla include: Cyanophyta (cyanobacteria), Glaucophyta, Rhodophyta (red algae), Chlorophyta (green algae), Euglenophyta (euglenoids), Dinophyta (dinoflagellates), Cryptophyta and Heterokontophyta. However, there is no general agreement among phycologists about the exact number of algal phyla (Sheath & Wehr 2015).

Algae are further classified into two subgroups, based on cell size and complexity: microalgae and macroalgae. Microalgae represent the majority of the algae and are microscopic unicellular organisms (with some colony-forming species), including eukaryotic and prokaryotic species. Macroalgae are eukaryotic multicellular organisms that resemble higher plants (Andersen 2013; Ge *et al*. 2016).

Most algae are found in aquatic environments, with microalgae being the most frequently algae detected in water (Bellinger & Sigee 2010), where they function as the primary producers in the food chain (Lee 2008). However, microalgae can be also found in a variety of terrestrial environments, including extreme environments such as snowfields, desert soil, hot springs and arctic environments (Delwiche 2007). They are also found in environments where they are exposed to extremes of pH, salt concentration and radiation (Seckbach & Oren 2007).

As a consequence of microalgal adaptation to extreme or stressed environments, these microorganisms exhibit metabolic changes that can interfere with the most vital functions of the cells, such as respiration, photosynthesis or cellular division, ultimately leading to cell survival

(Fogg 2001). Extreme environmental factors not only affect these basic cell functions, but also influence dynamic cell composition, as they intervene with the pattern, pathways and activities of cellular metabolism (Hu 2013).

The effect of environmental factors on microalgal cells leads cells to increasing the production of certain compounds, which creates obvious biotechnological consequences (Hu 2013). These compounds, usually the result of microalgal secondary metabolism, have made these microorganisms the target of investigation in different fields of biotechnology. Microalgae have been investigated as generators of green energy (Laamanen *et al*. 2016), as important sources in human and animal nutrition (Navarro *et al*. 2016a), as an incomparable food in aquaculture (Muller-Feuga 2013) and as sources of compounds of medical interest, such as with antiinflammatory, anticancer, antiviral, antifungal and antibacterial activities (Soontornchaiboon *et al.* 2012; Santoyo *et al.* 2012; Najdenski *et al.* 2013; Li *et al.* 2016). Although the number of studies on microalgal compounds for medical application has been increasing, microalgae are, however, still largely unexplored microorganisms in this field (Olaizola 2003).

1. 3 Microalgae and secondary metabolism

Secondary metabolism is the production of compounds that are not directly used by the cells for their growth or cell division (Carmichael 1992). Secondary metabolites are synonymously used with natural products (Maschek & Baker 2008) and include molecules that act as hormones, antibiotics, immunomodulatoring agents, pesticides, antitumor agents, growth promotors and toxins (Carmichael 1992; Demain 1998). While the primary metabolites are universal amongst microorganisms, the secondary metabolites are of restricted distribution, with

the biosynthetic capability tipically restricted to certain strains and conditions (Malik 1980; Shimizu 1996).

The biosynthetic pathways of secondary metabolites are higly regulated systems (Skulberg 2000). Under stress conditions, microorganisms reduce their growth rates and a pool of intermediates and end products of primary metabolism accumulate, and are used as precursors by specific pathway enzymes for the production of secondary metabolites (Malik 1980; Skulbert 2000). The precursors, usually amino acids, appear to stimulate the production of secondary metabolites when in increased supply and/or by inducing the expression of biosynthetic enzymes (Demain 1998). These enzymes are usually coded by clustered genes on chromosomal DNA and therefore, gene regulation will have an important effect on secondary metabolite production (Demain 1998; Skulbert 2000).

Microalgae have the ability to produce a series of secondary metabolites with diverse chemical structure and physiological functions (Skulberg 2000), which are not found in higher plants (Shimizu 1996). Despite microalgal potential, not many species have been investigated and their secondary metabolites identified (Shimizu 1996). Among microalgae investigated, cyanobacteria and dinoflagellates appear to be the most studied for the production of secondary metabolites (Carmichael 1992; Shimizu 1996; Skulberg 2000). Green microalgae have been little investigated, which is most likely due to green macroalgae being known as the least prolific producers of natural products (Maschek & Baker 2008). However, studies investigating biological activities from extracts of green microalgae have shown an interesting ability of these microorganisms to produce unknown compounds that have antioxidant, anti-inflammatory, anticancer and antimicrobial activities (Gigova *et al.* 2011; Al-Wathnani *et al.* 2012; Garbayo *et*

al. 2012; Soontornchaiboon *et al.* 2012). Therefore, these microorganisms seem to have the potential to harbour compounds of medical interest that may lead to new needed drugs.

1. 4 Microalgae and stress factors

1.4.1 Stress

Early knowledge on biological stress from unfavourable environments is based on the theories of Hans Selye, an endocrinologist from the Institute of Experimental Medicine and Surgery at McGill University. He studied the non-specific effect of stressors on humans. According to him, stress was the manifestation of a typical syndrome as a response to damage to the organism by non-specific agents, such as cold, surgical injury, excessive muscular exercise or intoxication with sub-lethal doses of drugs (Selye 1936).

According to Fogg (2001), who investigated algae, stress is an inherited characteristic of the organism to respond to small or large stimulus from the environment that affects its biological processes. Davison & Pearson (1996) divided stress on algae into two different types: the limitation stress, a damage caused due to inadequate supply of resources, and the disruptive stress, where damage occurs because of adverse conditions (or the allocation of resources to prevent damage). Stress on microalgae has been further classified as physical, nutritional and biochemical (Fogg 2001). Examples of physical stress may include osmotic stress, desiccation and high/low temperatures. Nutritional stress includes the deficiency of essential nutrients and biochemical stress may include low pH and high concentration of metals (Fogg 2001). Furthermore, biotic stress factors, such as competition and predation, can also interfere with microalgal metabolism, leading to production of active compounds (Leflaive & Ten-Hage 2007).

Studies have shown that microalgae from competitive habitats produce compounds of medical interest (Lustigman *et al.* 1988).

The application of physical, nutritional and/or biochemical stresses on microalgae have shown to trigger the overproduction of commercially valuable constituents such as lipid for biodiesel production (Han *et al.* 2016) and carotenoids (Minhas *et al.* 2016) as food additive in the nutraceutical industry. Furthermore, microalgae collected from stressed environments, either natural or anthropogenic, that exhibit high temperature or low pH have shown the ability to produce compounds of medical interest (Al-Wathnani *et al.* 2012; Navarro *et al.* 2016b).

Although different stress factors have shown to interfere with microalgae metabolism and activate the production of compounds of medical interest, this current study focuses particularly on abiotic stresses including low pH and high metal concentrations.

1.4.2 Low pH

Low pH in freshwater is usually associated with geothermal hot springs and mining activities (Fogg 2001). In mining, acid mine drainage (AMD) can create extremely low pH waters, usually a result of sulphides in rock strata becoming exposed to water and oxygen (Novis & Harding 2007). This phenomenon is usually the cause of low pH found in water bodies near mines (Novis & Harding 2007) and its main consequence is the disruption of the ecosystem with the elimination of sensitive species (Gray 1997).

Any microalgae that are present in acid mine drainage have to thrive in a usually oligotrophic environment, where nutrients, particularly inorganic nitrogen and carbon, are scarce (Novis & Harding 2007). Most of the microorganisms present in such extreme environments are

prokaryotes, but eukaryotic microalgae have been also documented as being metabolic active in extremely acidic environments (Nancucheo & Johnson 2012).

In order to survive in acidic pH environments, microalgae have to confront high H^+ concentrations (Gross 2000). As many enzymes are highly dependent on pH, microalgae have to be able to maintain a neutral cytosolic pH (Gerloff-Elias *et al*. 2005). Therefore, these microorganisms are expected to demonstrate important adaptation changes, such as low conductance of the plasma membrane for H^+, a high H^+ capacity and a high buffer capacity (Gimmler 2001). However, extreme pH conditions are known to exert an important influence on photosynthesis, growth and nutrient assimilation (Gerloff-Elias *et al*. 2005). As acidophilic microalgae present effective mechanisms to avoid the entrance of H^+, important cations required for growth, such as K^+ and NH_4^+, are also prevented from entering the cells (Gimmler 2001).

The presence of freshwater green microalgae in areas of low pH have been observed (Stokes *et al.* 1973; Aguilera *et al.* 2007). The biotechnological potential of these microorganisms, thriving in low pH environments, has been investigated for their ability to store triacylglycerols for biodiesel production (Eibl *et al.* 2014; Hirooka *et al.* 2014; Ruiz-Dominguez *et al.* 2015). However, studies on the biological activities of microalgae from low pH environments are still scarce (Ruiz-Dominguez *et al.* 2015; Navarro *et al.* 2016b). Therefore, the ability of these microorganisms, from acidic environments, to produce compounds of medical interest, particularly with antibacterial properties, is still to be determined.

1.4.3 High metal concentration

The ability of microalgae to survive in environments highly contaminated with heavy metals has been the focus of much investigation, in particular because of their bioremediation capacity (Monteiro & Castro 2012). Although the disposal of industrial wastes may expose microalgae to heavy metal contamination, water bodies affected by AMD are particularly prone to metal contamination, particularly where coal, pyritic sulphur, copper, zinc, silver and lead are mined (Gaur & Rai 2001; Gray 1997). Moreover, water bodies that have been contaminated with AMD have usually high concentrations of metals since the low pH is known to promote metal solubility in water (Gaur & Rai 2001; Novis & Harding 2007).

Certain metals such as copper, iron, zinc and manganese, in small concentrations, are necessary for microalgal to perform important cell functions, such as photosynthesis, phosphorus acquisition and DNA transcription (Miazek *et al.* 2015). However, microalgae growing in the presence of high concentrations of metals have shown characteristics of toxicity including reduction in growth and photosynthesis, inhibition of enzymes activity, alteration in protein structure and membrane integrity (Monteiro & Castro 2012).

Microalgae are known to be able to survive short periods of environmental stresses due to physiological acclimatisation through the modification of gene expression. However, when the extreme conditions exceed physiological limits, only mutations that confer resistance will enable microalgal adaptation (Garcia-Balboa *et al.* 2013).

The ability of microalgae to survive in areas of high metal contamination has been associated to a series of mechanisms of resistance, including the increased production of

extracellular polymeric substances that prevent metals getting to the cytoplasm (Novis & Harding 2007). The accumulation of metals inside the thylakoids (Novis & Harding 2007), the formation of metal complexes with excreted metabolites, activation of efflux pumps and elimination of a toxic metal through vaporization, by converting the metal into a volatile chemical species (Monteiro & Castro 2012) can all protect microalgae.

Heavy metal toxicity affects the size, diversity, activity and genetic structure of microorganisms, leading to changes in morphology, metabolism and growth (Ayangbenro & Babalola 2017). Microalgae under metal stress have shown generation of reactive oxygen species, with a common example being peroxides and the consequent disruption of the plasma membrane by lipid peroxidation (Novis & Harding 2007). Moreover, microalgal cells may also respond to metal induced oxidative stress by producing chelating and antioxidant agents, and enzymes that quench reactive oxygen species (ROS) (Miazek *et al.* 2015).

Interestingly, the evaluation of the *Chlamydomonas eustigma* genome, a green microalga adapted to environments with low pH and high metal concentrations, showed a series of genetic changes, including highly expressed genes of heat-shock proteins and plasma membrane H^+ - ATPase, the loss of fermentation pathways responsible for acidifying the cytosol, energy shuttle and buffering system, as well as arsenic detoxification genes in order to adapt to these stressed environments (Hirooka *et al.* 2017).

As microalgae adapt to environments with high concentrations of metals, metabolic adaptations will also occur (Nishikawa *et al.* 2003), which may lead to the production of compounds with biological functions (Garbayo *et al.* 2012). Although microalgae obtained from areas of high metal pollution have shown the ability to produce biologically active compounds

with antioxidant capacity (Garbayo *et al.* 2012), the ability of microalgae from these areas to produce active compounds with antibacterial activity has not been examined.

CHAPTER 2: THESIS HYPOTHESIS AND OBJECTIVES

Microalgae constitute a vast untapped source of compounds with biological activities, therefore these microorganisms represent a potential resource for investigation into compounds with antibacterial activity. Previous studies investigating the production of antibacterial compounds by microalgae have mainly focused on cyanobacteria, with relatively little attention to green microalgae. Extracts from green microalgae have shown the ability to inhibit human pathogens; however, the strains evaluated are from non-stressed environments, including laboratory strains. Microalgae exposed to environmental stresses, on the other hand, are known to produce secondary metabolites: the primary source of existing microbial antibiotic compounds. However, the ability of green microalgae, thriving in hash environments, under a combination of stress factors to exhibit antibacterial activity against human pathogens has not been determined.

2.1 HYPOTHESIS

Freshwater green microalgae surviving in areas near abandoned mine sites, where high metal concentrations and low pH have been present for many decades, should have undergone adaptations that could lead to production of metabolites exhibiting antibacterial activity. This would make these organisms a potential new natural source of antibiotics.

2.2 OBJECTIVES

The main objective of this study was to evaluate freshwater green microalgae from areas near abandoned mine sites in Northern Ontario, Canada, for antibacterial activity. The specific objectives were to:

1) isolate green microalgae from water bodies near abandoned mine sites in Ontario;

2) evaluate the *in vitro* susceptibility of Gram-positive and Gram-negative bacteria to methanolic extracts from these microalgae;

3) determine the point during the microalgal growth cycle that the highest antibacterial activity of extracts occurs;

4) assess the *in vitro* effect of extracts that exhibit antibacterial activity on mammalian cells viability.

CHAPTER 3: CYANOBACTERIA AND EUKARYOTIC MICROALGAE AS POTENTIAL SOURCES OF ANTIBIOTICS

(Review)

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3.1 ABSTRACT

Bacterial resistance to antibiotics is an issue receiving significant attention and highlights the urgent need for new classes of antibiotics. This has led to exploration for natural compounds from relatively unexplored microorganisms including microalgae. These photosynthetic organisms are extremely diverse and ubiquitous and have been shown to produce compounds that exhibit antibacterial activity against human pathogens. Their ability to survive in a wide-range of environmental conditions and the possibility of culture condition manipulation for optimization of antibacterial compounds suggests that they have significant potential. However, only a few species have been so far investigated and very few metabolites characterized. This review provides an overview of this research and illustrates the opportunity for significantly more systematic investigation of these microorganisms.

Keywords: Antibacterial activity, Bacteria, Metabolites, Natural compounds.

3.2 INTRODUCTION

An increase in bacterial resistance to existing antibiotics, which have been mostly obtained from bacteria, has encouraged the use of different technologies to find new efficient compounds. This has included the production of semi-synthetic and synthetic antibiotics (Fernandes 2006). However, increasing resistance to these compounds as well as the lack of new antibiotics highlight the need for continuous investigation to find new molecules and metabolic targets (Clardy *et al.* 2006). One successful approach that is being looked at again is the investigation of natural compounds, especially from untapped sources (Sheridan 2006).

Natural compounds are known for a wide range of structural diversities and pharmacological activities (Harvey 2000) that are not seen in synthetic antibiotics (Fernandes 2006). The recent exploration of alternative sources for these compounds has emphasized their potential for producing effective molecules against human pathogens (Jang *et al*. 2013; King *et al*. 2014; Ling *et al*. 2015).

This review focuses on investigation into the antibacterial activity of cyanobacteria and eukaryotic unicellular algae, microorganisms where photosynthesis is associated with chlorophyll *a*. Collectively, these microorganisms will be referred to as microalgae.

Photosynthetic microorganisms represent a potential source of antibiotic molecules, in particular secondary metabolites with chemical structures of low molecular weight less than 3000 daltons (Hernandez-Carlos & Gamboa-Angulo 2011). These compounds, which are produced in a variety of concentrations, depending upon environmental conditions, have the potential to affect other organisms (Skulberg 2000; Leflaive & Ten-Hage 2007).

The number of reported assessments of microalgae as sources of antibacterial compounds is considerably lower compared to other organisms, such as bacteria and fungi (Clardy *et al*. 2006). However, the need for new antibiotics has stimulated an increase in work focused on extracting and testing natural compounds from alternative sources, including microalgae. Furthermore, advances in technologies for identification and purification of small amounts of bioactive compounds offer more precise and faster ways to screen alternative natural sources (Gil-Chavez *et al*. 2013).

Screening programs have been established to find microalgal strains containing potentially useful secondary metabolites (Ordog *et al*. 2004; Plaza *et al*. 2010). Although the results look very promising, there are still no commercially available antibiotics from microalgae. This is likely due in part to the paucity of information regarding activity and toxicity *in vivo* (Borowitzka 1995), as well as the challenges of controlled large-scale production (Skulberg 2000; Olaizola 2003; Pulz & Gross 2004; Wijffels 2008; Skjånes *et al*. 2013).

Metabolites from microalgae are extremely diverse, with some of them being successfully commercialized (Spolaore *et al*. 2006; Borowitzka 2013). These microorganisms produce a wide range of chemical classes including alkaloids, indoles, macrolides, peptides, terpenes, acetogenins, phenols, fatty acids and volatile halogenated hydrocarbons (Amaro *et al*. 2011). A few of these have been associated with growth inhibition of pathogenic microorganisms (Hernandez-Carlos & Gamboa-Angulo 2011).

Microalgae offer interesting advantages as potential producers of antibiotics: they are extremely evolutionary and phylogenetically diverse (Borowitzka 1995); they can grow in bioreactors on a large-scale (Spolaore *et al*. 2006) and in inexpensive media (Pratt *et al*. 1944);

they can be stored by cryopreservation (Borowitzka 2013), and are able to produce a wide range of valuable organic compounds that can be manipulated by environmental changes (Andersen 2013).

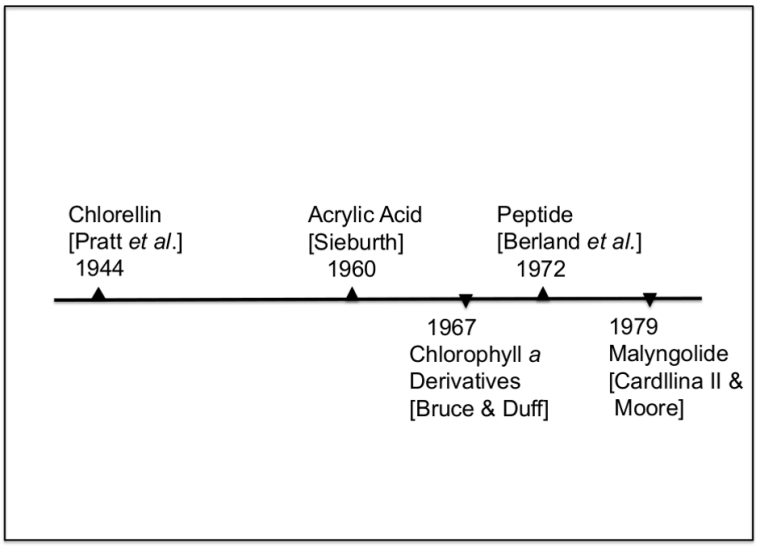


Figure 3.1. Important studies between 1944 and 1979 that found antibacterial compounds from microalgae.

3.2.1 Investigation of antibacterial activity of microalgae - an overview

The use of microalgae as medicine occurred well before systematic scientific research began (Hoppe 1979). The first reported observation of "toxic substances" secreted by microalgae was by Harder (1917) who worked with the cyanobacterium, *Nostoc punctiforme* (Kützing) Hariot (as *Nostoc commune* Vaucher *ex* Bornet & Flahault). Following on from this work there have been a few studies focused on agents excreted by algae cultured in colonies. The substances were described as being able to produce an auto-inhibitory effect as well as eliminate bacteria (Lefevre 1964).

Pratt *et al.* (1944) first isolated active compounds from microalgae. A mixture of fatty acids (chlorellin) from species of *Chlorella* showed *in vitro* inhibitory activity against both Gram-positive and Gram-negative bacteria. Interestingly, Pratt *et al*. (1951) described a practical application during World War II derived from the previous observations. According to them open sewage from the military installations, which had been heavily inoculated with *Chlorella* sp. was bacteriologically safe for discharge into local streams. It was noted that there was a reduction in the number of coliforms compared to areas where *Chlorella* sp. were not present (Pratt *et al*. 1951).

In the 1950s, systematic screening of algae for antibiotics began (Borowitzka 1995). However, over the subsequent decades the studies would focus mostly on macroalgae (seaweeds) (Mautner *et al*. 1953; Glombitza 1969; Reichelt & Borowitzka 1984). The use of these organisms as the main research targets was most likely due to their ready availability (Duff *et al*. 1966), as well as already being a source of many bioproducts, including food, agar, alginates and iodine (Pratt *et al*. 1951).

New observations on antibacterial activity of microalgae and potential antibacterial compounds slowly emerged through the 1950s to 1970s (Figure 3.1). The majority of these studies focused on the role of antibacterial substances in ecological interactions (Sieburth 1959; Burkholder *et al.* 1960; Jorgensen & Nielsen 1961; Duff *et al*. 1966; Bruce *et al*. 1967; Ramamurthy 1970; Berland *et al*. 1972). For example, Sieburth (1959) observed that a microalgal species from the Antarctic was able to release substances into the medium that reduced bacterial growth [*Escherichia coli* Migula (Castellani & Chalmers)], as well as modified the gastrointestinal flora of penguins that ingested the microalgae. In the following year, the same

researcher identified the microalga, a species of *Phaeocystis*, and isolated a volatile chemical (an acrylic acid) as being responsible for the antibacterial activity (Sieburth 1960).

Although the compound described by Sieburth (1960) showed promise as a broad-spectrum antimicrobial, the minimal inhibitory concentration was relatively high compared to known antibiotics, and acrylic acid *in vivo* demonstrated potential toxicity (Sieburth 1961). Similarly, in 1970, Ramamurthy evaluated the potential of the cyanobacterium *Trichodesmium erythraeum* Ehrenberg *ex* Gomont for antibacterial activity in the gastrointestinal contents of the sea gull *Laurus brunicephalus* Jerdon. Although no compounds were identified, it was observed that *T. erythraeum* obtained from the gull guts was able to inhibit *in vitro* growth of both marine and terrestrial bacteria (Ramamurthy 1970).

Despite the knowledge that microalgae are able to produce antimicrobial substances, in the 1960s very little was known regarding the nature, formation and action of the antibacterial compounds produced (Jorgensen & Nielsen 1961). Burkholder *et al.* (1960) observed that cell extracts of the dinoflagellate, *Gonyaulax tamarensis* Lebour [as *Alexandrium tamarense* (Lebour) Balech], were able to inhibit growth of *Staphylococcus aureus* Rosenbach but also promote the growth of a marine bacterium. Similarly, culture filtrates of *Chlorella vulgaris* Beijerinck showed ability to inhibit but also accelerate growth of *S. aureus* (Jorgensen & Nielsen 1961). Although light seemed to play a role, factors and conditions influencing these behaviors were unknown.

During the 1960s and 1970s bacterial resistance to existing antibiotics became an important issue, adding urgency to the search for new compounds and new metabolic targets (Fernandes 2006). Antibiotic analogs (*e.g.* penicillin and cephalosporin derivatives) started to be synthesized in an effort to combat bacterial resistance (Brumfitt & Hamilton-Miller 1988). Duff *et al*. (1966)

were among the first to emphasize the need to investigate antibiotic metabolites from microalgae for therapeutic purposes. They assessed organic extracts from a wide panel of marine microalgae *in vitro* against marine and terrestrial bacterial strains. Although they saw selective action against bacterial strains, they also noticed the presence of bacterial resistance to crude extracts.

In 1967, Bruce and collaborators reported isolation of two chlorophyll *a* derivatives from the previously investigated microalga, *Isochrysis galbana* Parke. When they tested these compounds against bacteria, they noticed similarities to previous results and suggested that the two chlorophyll *a* derivatives were actively responsible for antibiotic activity.

Berland *et al.* (1972) isolated a peptide from the culture filtrate of *Stichochrysis immobilis* Pringsheim, which showed antibacterial activity against marine bacteria. Although their interest was to understand the importance of this activity in ecological interaction, the results showed the ability of the compound to inhibit bacteria from different origins. Similarly, in 1979, malyngolide, an antibiotic compound from the cyanobacterium *Lyngbya majuscula* Harvey *ex* Gomont was isolated and characterized after shown to be effective against *Mycobacterium smegmatis* (Trevisan) Lehmann & Neumann and *Streptococcus pyogenes* Rosenbach (Cardllina II *et al.* 1979).

In the 1980s, four decades after Pratt *et al*. (1944) isolated the first antimicrobial compound from microalgae, screening of photosynthetic microorganisms for antibiotics became more common. Cyanobacteria have been the major investigated photosynthetic microorganisms (Bloor & England 1989; Jaki *et al.* 1999; Ghasemi *et* al. 2003; Ghasemi *et al*. 2004; Soltani *et al*. 2005; Volk & Furkert 2006; Parisi *et al.* 2009; Kumar *et al*. 2011).

3.2.2 Antibacterial activity of cyanobacteria

Cyanobacteria are photoautotrophic organisms with a classic prokaryotic cell organization, but similarly to eukaryotes conduct photosynthesis and respiration in their active membrane system (Singh *et al*. 2011). They grow ubiquitously and produce in addition to toxins a wide range of bioactive metabolites (Bloor & England 1989), with potential application in biotechnology. These characteristics have made them focus of intense investigation in the last decade (Dahms *et al.* 2006; Wijffels *et al*. 2013).

To date, only a few compounds have been extracted and commercialized, including nutraceuticals, cosmetic products, and other high value molecules (Spolaore *et al*. 2006; Borowitzka 2013). Some purified compounds have promising commercial applications as bioplastics, biofertilizers, antiviral, antifungal, anticancer and antibacterial drugs (Patterson *et al*. 1994; Abed 2009; Leao *et al*. 2013).

Lipophilic and hydrophilic extracts from cyanobacteria collected in different areas of the globe have been evaluated for their antibacterial activities in cell-based *in vitro* assays. In most of the studies bioassays have been performed using crude extracts, which have indicated that species collected from different habitats (*e.g*. soil, marine and freshwater environments) are rich sources of inhibitory substances against Gram-positive and Gram-negative bacteria (Volk & Furkert 2006; Madhumathi *et al.* 2011; Prakash *et al*. 2011; Abdo *et al.* 2012; Kumar *et al*. 2012; Thummajitsakul *et al*. 2012; Yadav *et al*. 2012; Najdenski *et al*. 2013; Tiwari & Sharma 2013; Abazari *et al*. 2013; Mudimu *et al.* 2014).

Although most studies on antibacterial activity of cyanobacteria follow standard methodologies, a few others lack important information such as crude extract concentration

and/or a standardized bacterial concentration tested. Another omission includes the lack of solvent controls, which is essential as bacteria can be sensitive to the organic solvent used to recover the extract, therefore giving a potential false positive result (Fernandes 2003). Moreover, important missing data may include culture purity (axenic or non-axenic), which helps identify the species to be able to synthesize the effective compound (Borowitzka 1995). Similarly important is information regarding replicability, since it is not uncommon for natural sources to lose their ability to produce the compounds of interest (Wright 2014), as experienced by Ploutno & Carmeli (2000) working with *Nostoc* sp. (TAU-IL-220-1).

Other important information to be determined when evaluating extracts for antibacterial activity is the concentration able to inhibit or kill bacteria. In particular, extracts showing minimum inhibitory concentrations (MIC) below the MIC standardized by the Clinical and Laboratory Standards Institute (CLSI) should be further investigated (Silver 2011). Furthermore, it is of great importance that evaluations of *in vivo* activity follow isolation of compounds, since it is unknown if the effect of such molecules *in vivo* will mimic the results found *in vitro.*

A few studies have shown the *in vitro* effectiveness of purified and structurally identified compounds from cyanobacteria against bacteria. Among the bioactive compounds found is ambigol A from *Fischerella ambigua* (Kützing *ex* Bornet & Flahault) Gomont (EAWAG 108b), described as having potent activity against *Bacillus subtilis* (Ehrenberg) Cohn (Falch *et al.* 1993). Kawaguchipeptin B was extracted from *Microcystis aeruginosa* (Kützing) Kützing (NIES-88) and showed inhibition of *S. aureus* at a MIC of 1 μg/ml (Ishida *et al.* 1997). Noscomin is an extracellular diterpenoid produced by *N. commune* (EAWAG 122b) that showed inhibition of *Bacillus cereus* Frankland & Frankland (MIC 32 ppm), *S. aureus* (MIC 8 ppm) and *E. coli* (MIC 128 ppm) (Jaki *et al.* 1999). A different diterpenoid was also extracted from *N. commune*

(EAWAG 122b) and exhibited potent antibacterial activity against *Staphylococcus epidermidis* (Winslow & Winslow) Evans (MIC 4 ppm) (Jaki *et al.* 2000).

Other compounds extracted from cyanobacteria include coriolic acid, alpha-dimorphecolic acid and linoleic acid, from *Oscillatoria redekei* van Goor [as *Pseudanabaena redeckei* (van Goor) B.A.Whitton] (HUB 051), which inhibited the growth of *S. aureus* strains (Mundt *et al*. 2003). Nostocyclyne, from *Nostoc* sp. (TAU IL-220-1), is a cyclophane that was able to inhibit the growth of *S. aureus* and *B. subtilis* (Ploutno & Carmeli 2000). Furthermore, pahayokolide A from *Lyngbya* sp. was able to inhibit both *Bacillus megaterium* de Bary and *B. subtilis* at a MIC of 5 μg/ml, but showed toxicity to zebrafish embryos (LC_{50} 2.15 μM) (Berry *et al*. 2004). Gamma-linolenic acid from *Fischerella* sp. inhibited *S. aureus* (ATCC 25923) (MIC 4 μg/ml) (Asthana *et al*. 2006) whilst ambiguine I isonitrile, extracted from *Fischerella* sp. (TAU IL-199-3-1) inhibited *B. subtilis* (MIC 0.312 μg/ml) and *Staphylococcus albus* Welch (as *S. epidermidis*) (MIC 0.078 μg/ml) (Raveh & Carmeli 2007). Additionally, ambiguine I isonitrile inhibited *S. aureus* (MIC 8.9 μM), hapalindole G inhibited *Mycobacterium tuberculosis* (Zopf) Lehmann & Neumann (MIC 6.8 μM) and hapalindole H inhibited *Mycobacterium smegmatis* (Trevisan) Lehmann & Neumann (MIC 39.6 μM). All three compounds were extracted from *F. ambigua* (UTEX 1903) and showed low toxicity (IC_{50} > 128μM) against green monkey kidney cells (Mo *et al.* 2009a). Scytoscalarol was extracted from *Scytonema* sp. (UTEX 1163) and showed activity against *S. aureus* (MIC 2 μM), *Bacillus anthracis* Cohn (MIC 6 μM), and *M. tuberculosis* (MIC 110 μM). Moreover, scytoscalarol exhibited low toxicity (IC_{50} 135 μM) against green monkey kidney cells (Mo *et al.* 2009b). A new alkaloid (fischambiguine B) was extracted from *F. ambigua* (UTEX 1903) and had inhibitory activity against *M. tuberculosis* (MIC 2 μM), *S aureus* (MIC 19.4 μM), and *M. smegmatis* (MIC 23.4 μM) (Mo *et al.* 2010). Furthermore,

fischambiguine B exhibited low toxicity (IC_{50} > 128μM) against green monkey kidney cells (Mo *et al.* 2010). Table 3.1 illustrates some antibacterial compounds extracted from cyanobacteria.

Table 3.1. Antibacterial compounds extracted from cyanobacteria.

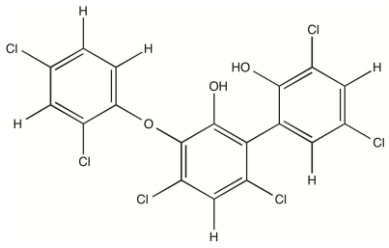
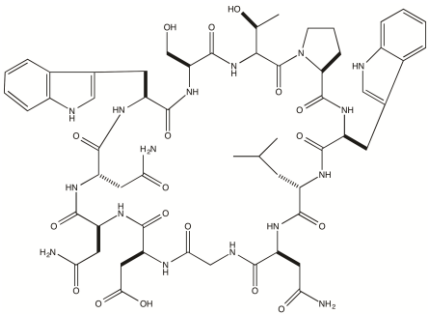
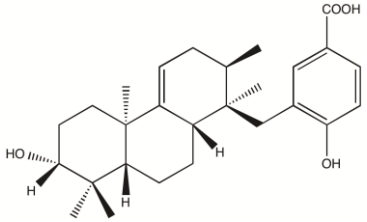
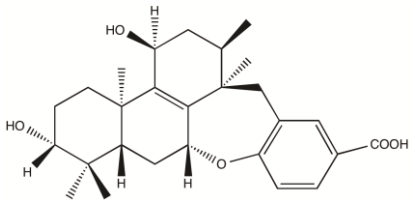
Species	Compound	Chemical structure	Reference
Fischerella ambigua	ambigol A		Falch *et al.* 1993
Microcystis aeruginosa	kawaguchipeptin B		Ishida *et al.* 1997
Nostoc commune	noscomin		Jaki *et al.* 1999
Nostoc commune	diterpenoid		Jaki *et al.* 2000

Table 3.1. (continued).

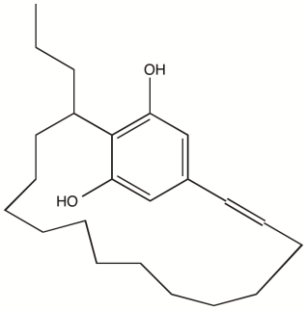
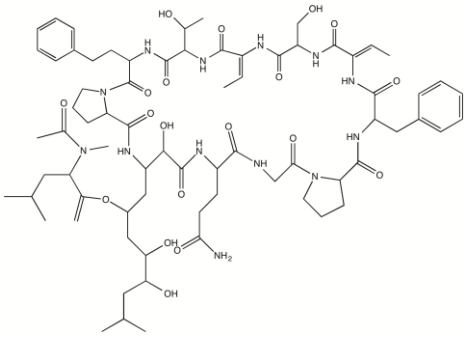
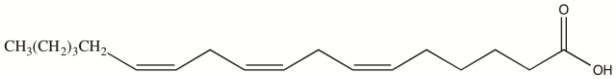
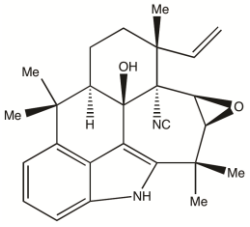
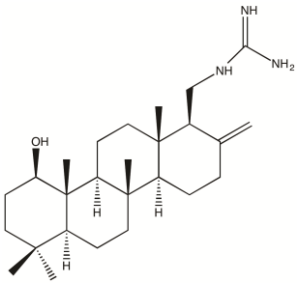
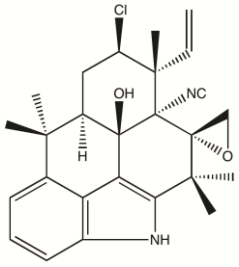
Species	Compound	Chemical structure	Reference
Nostoc sp.	nostocycline A		Ploutno & Carmeli 2000
Lyngbya sp.	pahayokolide A		Berry *et al.* 2004
Fischerella sp.	γ-linolenic acid		Asthana *et al.* 2006
Fischerella sp. *F. ambigua*	ambiguine I isonitrile		Raveh & Carmeli 2007 Mo *et al.* 2009a

Table 3.1. (continued).

Species	Compound	Chemical structure	Reference
Scytonema sp.	scytoscalarol		Mo *et al*. 2009b
Fischerella ambigua	fischambiguine B		Mo *et al*. 2010

Many bioactive metabolites synthesized by cyanobacteria are a peptide, a macrolide, a combination of peptides and macrolides, or belong to the alkaloid class (Mandal & Rath 2015). Even though these classes of compounds are produced by other microorganisms, including the antibiotic-producing actinomycetes (Chaudhary *et al.* 2013), novel antibacterial metabolites are synthesized by cyanobacteria (Ploutno & Carmeli 2000; Mo *et al.* 2009a), which suggests these microorganisms are worthy of further investigation.

3.2.3 Antibacterial activity of eukaryotic microalgae

Eukaryotic microalgae are highly diverse organisms able to synthesize a series of bioactive compounds such as isoprenoids, polyketides, nonribosomal peptides, polyunsaturated fatty acids, and alkaloids, which makes them commercially interesting (Sasso *et al*. 2012). Further studies have identified fatty acids, terpenes, carbohydrates, glycolipids, lipoproteins, bromophenols and tannins that exhibit antibacterial activity against human pathogens (Stein & Borden 1984; Metting & Pyne 1986). However, little is known about these compounds. Remaining questions include under what conditions the compounds are produced and their synthesis enhanced, what bacterial species are most sensitive and how (bactericidal or bacteriostatic) and where they act on bacteria.

It is known that microalgae accumulate cell-associated antibacterial substances (Cooper *et al*. 1983; Cannell *et al*. 1988). However, studies have shown different levels of antibacterial activity in microalgal culture filtrate, suggesting that microalgal cells excrete these substances, which may depend on the stage of growth and/or the species/strain evaluated (Pratt *et al*. 1944; Hansen 1973; Cooper *et al*. 1983; Kokou *et al*. 2012).

A limited number of studies have tested cell extracts for antibacterial activity using organic and inorganic solvents. Of the studies testing both lipophilic and hydrophilic extracts against bacteria, most have shown better results from lipophilic extracts obtained with solvents of different polarities (Ordog *et al*. 2004; Nair & Krushnika 2011; Seraspe *et al*. 2012). This suggests a lipophilic nature of the antibacterial compounds. However, in fewer studies, aqueous extracts showed broader antibacterial results, suggesting that the specific strain or harvesting time may play a role on the nature of the extracted compounds (Cooper *et al*. 1983; Nair & Krushnika 2011).

Crude extracts from different species of eukaryotic microalgae have shown effectiveness against both Gram-positive and Gram-negative bacteria, as well as *M. tuberculosis* (Prakash & Bhimba 2005; Desbois *et al*. 2009; Arun *et al*. 2012; Bai & Krishnakumar 2013; Danyal *et al*. 2013). This could suggest their potential for producing compounds with broad-spectrum activity, which is highly desired for new antibiotics. On the other hand, some studies have shown a specific inhibitory activity against Gram-positive bacteria (Debro & Ward 1979; Cannel *et al*. 1988), including methicillin-resistant *S. aureus* (MRSA) (Ohta *et al*. 1994; Ohta *et al*. 1995). These are interesting findings, considering the urgent need for effective antibiotics against Gram-positive organisms such as methicillin-resistant staphylococci, penicillin- and erythromycin-resistant pneumococci and vancomycin-resistant enterococci (Aksoy & Unal 2008). However, the numbers of studies focusing on isolation and identification of these metabolites is still quite low. This is likely to be due to greater interest in other microorganisms, in particular actinomycetes that are already known to produce antibiotics. Moreover, further investigations including toxicity, compound bioavailability, and *in vivo* effectiveness are needed.

Among the antibacterial compounds that have been identified from eukaryotic microalgae are acrylic acid (Sieburth 1960), fatty acids (Pratt *et al*. 1944; Findlay & Patil 1984; Ohta *et al*. 1994; Desbois *et al*. 2008), and pigments such as carotenoids and chlorophylls and their derivatives (Bruce *et al*. 1967; Bhagavathy *et al*. 2011). The isolation and identification of compounds are extremely important to evaluate their novelty and effectiveness. However, it is likely that many compounds extracted from these organisms will be impractical as antibiotics for medical reasons, due to toxicity or inactivity *in vivo* (Borowitzka 1995). There may also be restrictions due to commercial reasons, such as the inability for chemical synthesis, as experienced by Pratt with chlorellin (Willis 2007). As a possible solution, Borowitzka (1995) suggested that these compounds could serve as lead molecules or find application in agriculture.

Despite antibacterial products from microalgae being promising, one of the main challenges is likely to be the small concentration of compounds extracted (Ohta *et al*. 1994). This makes it difficult to produce a natural antibiotic from these microorganisms in large-scale. Nonetheless, if the compound has a great potential as antibiotic, the possibility of chemical synthesis through improved technologies may represent a feasible option (Borowitzka 2013).

3.2.4 Factors and conditions affecting production of microalgal secondary metabolites exhibiting antibacterial activity

Secondary metabolites differ from primary metabolites in that they are non-ubiquitous molecules that are usually not involved with energy production, structure or reproduction. They represent a wide range of biomolecules with important properties that include antibacterial activity (Demain 1981; Burja *et al*. 2001; Maschek & Baker 2008).

Variations in temperature, light, pH, salinity and nutrient availability have been extensively investigated for their impact on microalgal growth and their primary metabolism. This includes lipid (triacylglycerols) accumulation and other high-value biomolecule accretion, such as polyunsaturated fatty acids (Sang *et al.* 2012; Skjånes *et al*. 2013; Juneja *et al*. 2013; Breuer *et al*. 2013). Secondary metabolites are similarly likely to be affected by environmental changes, as their production seems to be directly linked to primary metabolite pathways (Malik 1980; Burja *et al*. 2001).

The production of secondary metabolites by microalgae can help these organisms survive in adverse conditions (Leflaive & Ten-Hage 2007). It seems plausible, therefore, to assume that strains growing in extreme environments or under induced metabolic stress will produce secondary metabolites with antibacterial activity.

Lustigman *et al.* (1988) investigated extracts of *Dunaliella* spp. isolated from polluted versus clean waters. They noticed that a heat labile non-proteinous substance that inhibited *E. coli* was only produced by species from polluted water. It was, therefore, suggested that microalgae from highly competitive habitats are more likely to produce compounds with antibacterial activity.

Al-Wathnani *et al.* (2012) evaluated antibacterial activity of microalgae that survived extreme temperatures in desert soils. According to their study, extracts of selected strains of cyanobacteria strongly inhibited bacterial growth, especially the Gram-negative *Shigella sonnei* (Levine) Weldin. Similarly, cell extracts of cyanobacteria collected from a hot spring in Iran were especially effective against Gram-positive bacteria, with large zones of inhibition obtained (Heidari *et al*. 2012).

Production of secondary metabolites appears to be species and even strain dependent (Leflaive & Ten-Hage 2007), and possibly also associated with specific environmental conditions. Therefore, careful evaluation of growth phase and conditions for each strain is necessary when assessing antibacterial production. Debro & Ward (1979) noticed that harvesting time was directly related to antibacterial activity in some freshwater microalgae.

Cooper *et al*. (1983), who worked with diatoms, found a similar outcome. They observed that the cell growth phase at harvesting was a relevant factor when evaluating antibacterial activity, as higher activity was associated with the stationary phase.

Limiting nutrients, light control and temperature were also shown by Trick *et al*. (1984) to influence the extracellular concentrations of a norcarotenoid (β-diketone), an antibacterial compound released by the marine dinoflagellate, *Prorocentrum minimum* (Pavillard) J.Schiller [as *Prorocentrum cordatum* (Ostenfeld) J.D.Dodge].

Chetsumon *et al*. (1994) noticed that variations in CO_2 concentration, light intensity, and inoculum concentration were also important factors for *Scytonema* sp. to produce antibacterial compounds in a photobioreactor. Ohta *et al.* (1995) showed the influence of nutrient availability on the antibacterial activity of *Dunaliella primolecta* Butcher (C-525) and *Chlorococcum* sp. (HS-101) extracts against MRSA. Extracts of *D. primolecta* cells growing in medium with modified concentrations of magnesium sulfate and calcium chloride were associated with enhanced antibiotic activity. Additionally, pH was also considered a key factor for controlling antibacterial activity of *Synechococcus* leopoliensis (Raciborski) Komárek [as *Romeria leopoliensis* (Raciborski) Koczwara] against *S. aureus*. A broad pH range (pH 5-10) was

appropriate for cell growth but not necessarily for the production of the antibacterial agent (pH 7-9) (Noaman *et al*. 2004).

3.2.5 Techniques and technologies for the identification of antibacterial compounds from microalgae

Advances in technologies for detection, purification and identification of small amounts of molecules present in mixed extracts have increased opportunities for investigation of antibacterial compounds from natural products (Leeds *et al*. 2006). The search for alternative unexplored sources of natural compounds has also created a new perspective for finding new lead molecules or new antibiotics (Harvey 2000).

Most studies that tested antibacterial compounds from microalgae employed a biological screening approach, mostly *in vitro* antibacterial susceptibility testing, including agar diffusion and/or broth dilution techniques (Mo *et al*. 2009b; Seraspe *et al*. 2012). This approach allows selection of the most interesting strains and identification of important factors such as optimal growth conditions and selection of solvents used for extraction.

In antibacterial drug discovery, once biological activity is detected, the following steps involve separation of complex mixtures, purification and structural elucidation of the single biologically active compound (Leeds 2006). Reports on the purification and identification of antibacterial compounds from microalgae are still scarce in the literature. On the other hand, the recent increase in antibiotic research, the potential of these organisms to produce secondary metabolites, and the availability of analytical technologies such as chromatography and spectroscopy should help stimulate further investigation of antibacterial compounds from microalgae.

Chromatography is a useful technology that separates compounds based on a system containing one mobile and one stationary phase. Before the advent of chromatography, the purification of natural compounds was done by extraction of large amounts of starting material, and crystallization of the pure substance in a few crystals (Ettre 2000).

When Pratt *et al*. (1944) discovered chlorellin from species of *Chlorella* they did not have the wide chromatographic separation techniques available today, as partition (liquid-liquid) and paper chromatography were just being introduced (Martin *et al*. 1941; Consden *et al*. 1944). At that time, column chromatography was the best choice for those dealing with separation of natural compounds. However, this technique was in its early stages and relied particularly on the skill of the researcher to prepare the columns (Ettre 2000).

Gas-liquid chromatography was introduced by James & Martin (1952), initially for separation of fatty acids and then later expanded to other organic compounds. In two decades this technique became the most important analytical technique for natural product mixtures (Ettre 2000). Although it has an excellent separation capacity, its application is restricted to volatile samples (Marston & Hostettmann 2009), requiring extensive sample preparation to vaporize non-volatile matrices (Seger *et al.* 2013).

Porath & Flodin (1959) introduced the term "gel filtration" for fractionation of compounds using synthetic hydrophilic gel (dextran gel known as Sephadex) in column chromatography. In this technique, compounds were separated based on their molecular dimensions and the degree of cross-linkage of the gel (Porath & Flodin 1959). The technique was soon expanded to molecular size fractionation of hydrophobic macromolecules using polystyrene gel eluted with solvents, and its name changed to "gel permeation chromatography" (Moore 1964). These easy and

inexpensive techniques emerged and were named “size exclusion chromatography”, a branch of liquid chromatography (Ettre 2000). This has been used for initial fractionation of antibacterial compounds from microalgal extracts (Table 3.2).

When the first antibacterial compound (acrylic acid) from a microalga was isolated and identified (Sieburth 1960) paper chromatography and x-ray crystallography were used, and the unknown compound was compared to a commercially available acrylic acid.

The use of paper chromatography had the disadvantage of having slow migration rates, since only cellulose was available as the stationary phase matrix (Ettre 2000).

Thin layer chromatography (TLC) was proved (Stahl 1958) to produce much quicker analysis of multiple samples, as a result of the separation being performed on a porous layer consisting of small particles and of the use of a variety of stationary phases, as well as semiquantitative analysis (Ettre 2000; Marston & Hostettmann 2009).

Studies performing purification of antibacterial compounds from microalgae in 1970s and 1980s used mostly gel chromatography and TLC (Table 3.2). TLC is a simple and fast technique that generates an image as a result. However, the technique presented problems with automation, quantitation and reproducibility (Marston & Hostettmann 2009), which were solved by high performance liquid chromatography (HPLC), the most used analytical tool (Ettre 2000).

Following compound purification, structure elucidation is an essential step in drug discovery, allowing researchers to select new compounds and discard known ones, also called dereplication (Lang *et al*. 2008). Purified compounds from microalgae have been elucidated

(Table 3.2) by available spectroscopic techniques including mass spectrometry and nuclear magnetic resonance.

Mass spectrometry (MS) is an analytical tool widely used to identify and quantify natural products. Its main advantages are speed, sensitivity, selectivity and versatility in analyzing solids, liquids and gases (Hocart 2010). Nuclear magnetic resonance (NMR) has been considered essential to establish molecular structures by providing structural information, atomic connectivity and stereochemistry that MS cannot (Mandal & Rath 2015). However, NMR works best if the substance analyzed is highly pure, due to the signal richness of its spectra (Seger 2013).

Advances in these techniques have made possible the complete structure elucidation of natural compounds by using small amounts of pure compounds (Leeds *et al*. 2006). Studies focusing on secondary metabolites from microalgae have used more recently available analytical tools and obtained interesting results. The use of matrix assisted laser desorption ionization time of flight (MALDI-TOF) MS allowed the identification of new secondary metabolites in extracts of cyanobacteria. Environmental samples were evaluated for novel compounds prior to culturing, with high sensitivity and low sample volume (Puddick & Prinsep 2008). Furthermore, real time mass spectrometry (DART-MS) was used to identify *Nostoc* sp. based on chemical finger printing analysis. The method was suggested as a means of providing rapid identification of the major components of a species (Singh & Verma 2012).

Table 3.2. Selected antibacterial compounds obtained from microalgae from 1960.

Species	Compound(s)	Source	Method (s)	Reference
Phaeocystis sp.	acrylic acid	cells	paper C,[1] X-ray crystallography	Sieburth 1960
Stichochrysis imobilis	peptide	culture medium	gel C, TLC[2]	Berland *et al*. 1972
Lyngbya majuscula	δ-lactone malyngolide	cells	gel C, X-ray crystallography	Cardllina II *et al*. 1979
Navicula delognei[3]	ester	cells	gel C, TLC	Findlay & Patil 1984
Fischerella ambigua	ambigol A and B	cells	NPVLC,[4] MPLC,[5] R-P-HPLC,[6]EIMS,[7] HREIMS,[8]x-ray crystallography, NMR,[9] IR,[10] UV[11]	Falch *et al*. 1993
Chlorococcum sp. *Dunaliella primolecta*	α-linolenic acid	cells	gel C, TLC, GC[12] GC-MS,[13] NMR	Ohta *et al*. 1995
Microcystis aeruginosa	kawaguchipepeptin B	cells	flash C, R-P-HPLC, MPLC, UV	Ishida *et al*. 1997

Table 3.2. Continued.

Species	Compound(s)	Source	Method (s)	Reference
Nostoc commune	noscomin	culture medium	SPE,[14] gel C, HPLC, TLC, IR 2D[15] NMR, ESIMS,[16] UV	Jaki *et al*. 1999
Nostoc sp.	nostocycline A	cells	gel C, HPLC, HREIMS, 2D-NMR, IR	Ploutno & Carmeli 2000
Nostoc commune	diterpenoid anthraquinone indane derivative	cells	gel C, R-P-C, 2D-NMR, EIMS	Jaki *et al*. 2000
Oscillatoria redekei	fatty acids (coriolic acid and α-dimorphecolic acid	cells	gel C, TLC, HPLC, NMR, MS, UV	Mundt *et al*. 2003
Lyngbya sp.	pahayokolide A	cells	gel C, SPE, HPLC, R-P-HPLC	Berry *et al*. 2004

Table 3.2. Continued.

Species	Compound(s)	Source	Method (s)	Reference
Fischerella ambigua	parsiguine	culture medium	TLC, gel C, NMR, IR	Ghasemi *et al*. 2004
Fischerella sp.	fatty acid (γ-linolenic acid)	cells	TLC, gel C, HPLC	Asthana *et al*. 2006
Fischerella sp.	alkaloids (ambiguine H and I isonitriles)	cells	flash C, gel C, R-P-HPLC, NMR, IR, UV HREIMS	Raveh & Carmeli 2007
Scytonema sp.	scytoscalarol	cells	Column C, MS 2D-NMR,HRMS,[17] NMR	Mo *et al*. 2009b
Nostoc sp. CCC 537	diterpenoid	cells	TLC, R-P-HPLC NMR, ESIMS, EIMS, IR, UV	Asthana *et al.* 2009

[1] C, chromatography; [2] TLC, thin layer chromatography; [3] Navicula delognei f. elliptica Lobban [as Parlibellus delognei f. ellipticus (Lobban) E.J. Cox]; [4] NPVLC, normal-phase vacuum liquid chromatography; [5] MPLC, medium pressure liquid chromatography; [6] R-P-HPLC, reversed-phase high-performance liquid chromatography; [7] EIMS, electron-impact mass spectrometry; [8] HREIMS, high-resolution electron-impact mass spectrometry; [9] NMR, nuclear magnetic resonance; [10] IR, infrared; [11] UV, ultraviolet–visible; [12] GC, gas-chromatography; [13] MS, mass spectrometry; [14] SPE, solid-phase extraction; [15] 2D, two-dimensional; [16] ESIMS, electrospray ionization mass spectrometry; [17] HRMS, high-resolution mass spectrometry.

The processing and analysis of extracts leading in many cases to a known compound is still a challenge for antibacterial research (Lang *et al*. 2008). This situation has been improved through the use of hyphenated techniques (Wolfender *et al*. 2006). Hyphenated techniques combine both chromatographic and spectral methods exploiting the separation of compounds by chromatography and identification by spectroscopy (Patel *et al.* 2010). This combination allows researchers to determine the potential novelty of a compound based on both mass and elution time before large-scale purification and characterization (Puddick & Prinsep 2008). Some examples of widely used hyphenated techniques include gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), LC-NMR, and LC-NMR-MS.

The coupling of HPLC with MS (LC-MS) is very efficient in terms of detection, quantification and identification of a wide range of natural products. The direct coupling of HPLC with NMR can provide full structure elucidation and stereochemical information (Wolfender *et al.* 2010). Furthermore, the coupling of LC with MS or NMR enables separation of molecules preceding analysis, resulting in improved signal intensity. The combination of ultrahigh pressure liquid chromatography with time-of-flight MS detectors has shown to be very

effective for identification of new compounds and dereplication of natural product extracts (Mandal & Rath 2015).

The availability of advanced analytical techniques should have an important impact on antibacterial discovery. The requirement of only small amounts of samples and the ability to separate compounds from complex matrices should simplify the investigation of natural sources including microalgae. Table 3.2 summarizes techniques and technologies that have been used to purify and identify compounds from microalgae.

3.2.6 CONCLUSION

The diversity and ubiquity of microalgae, together with their ability to produce secondary metabolites exhibiting antibacterial activity, could make them an important source of new antibiotics. Significant value may lie in their ability to survive and adapt to a wide range of environments along with the products synthesized when their environmental conditions are changed or stressed. A wide range of technologies are available to aid systematic identification and purification of these natural products, which combined with *in vivo* experiments, could lead to novel antibiotics. However, to date there has been relatively little research into these microorganisms and they mainly remain an "untapped" source.

In summary:

1. The need for new antibiotics means microalgae that produce secondary metabolites that inhibit Gram-positive and/or Gram-negative bacteria should be more extensively investigated. That in part could include reviewing existing libraries of known microalgae metabolites and testing promising candidates for antimicrobial activity.

2. Changes in environmental conditions have been associated with enhancement of antibacterial compounds production by microalgae.

3. The ability of certain microalgal species to survive and adapt to extreme environmental conditions suggests that strains from these environments are particularly worthy of exploration for their potential to produce antibacterial compounds.

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CHAPTER 4: BIOPROSPECTING FRESHWATER MICROALGAE FOR ANTIBACTERIAL ACTIVITY FROM WATER BODIES ASSOCIATED WITH ABANDONED MINE SITES

(Original Research)

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4.1 ABSTRACT

Bacterial resistance to antibiotics necessitates the search for new sources of microorganisms able to produce these needed molecules, which are typically secondary metabolites produced as a protective mechanism. Microalgae can produce a wide-range of secondary metabolites as a response to environmental stress but have been the subject of little research as potential sources of antibiotics. As a step towards assessing their potential, we isolated 40 freshwater green microalgae from water bodies with a wide-range of metal concentrations and pH values that were near abandoned mine sites in northern Ontario, Canada. Microalgae from this region and these types of water bodies had not been previously investigated for antibacterial properties. Forty methanolic microalgal extracts were obtained, analyzed and tested against Gram-positive and Gram-negative bacteria, and 37.5% inhibited the human pathogen *Staphylococcus aureus* (Bacilli). This is a higher "hit-rate" than in previously published results, and furthermore, the minimum inhibitory concentrations against *S. aureus* were notably much lower than any other reported work. This is the first time such environments have been assessed, and whilst no clear association was observed between the metals and pH analyzed, and antibacterial activity, the findings do indicate that microalgae from anthropogenically stressed environments are a potential source of antibacterial compounds. That is, sites that are typically regarded as having no value and often very negatively perceived are potential sources of valuable bioactive compounds. Therefore, future studies are necessary to determine what environmental thresholds are associated with the antibacterial activity of the freshwater green microalgae thriving in these environments.

Keywords: Antibiotics, Antimicrobial, Metabolites, Pathogens, Stressed environments

4.2 INTRODUCTION

The discovery of penicillin in the late 1920s and other antibiotics that followed represents the major scientific achievement against deadly bacterial infections (Fernandes 2006). Certain bacteria from soil and fungi have been successfully providing many antibacterial drugs (Lam 2007). This was possibly due to these microorganisms producing secondary metabolites (Demain 1981; Burja *et al*. 2001) that provide important mechanisms in ecological interactions (Leflaive & Ten-Hage 2007). However, the decrease in the effectiveness of existing antibiotics is necessitating research to look for unexplored natural sources (Jang *et al.* 2013; Stanton 2013; Ling *et al.* 2015).

Microorganisms for potential investigation are the relatively little-explored microalgae (Mudimu *et al.* 2014; Patel *et al.* 2015). Due to their diversity and adaptability, microalgae are found in almost every ecosystem, including fresh and marine water, rocks and extreme environments such as desert soils, snow and hot springs (Lee 2008). Furthermore, microalgae have the ability to produce metabolites, which have garnered considerable interest as a potential source of biofuels (Wijffels *et al.* 2013; Eibl *et al.* 2014) and for bioactivities, including antioxidant, anticancer and to a lesser extent, antibacterial activity (Ghasemi *et al.* 2007; Bhagavathy *et al*. 2011; Gigova *et al.* 2011; Al-Wathnani *et al.* 2012; Goiris *et* al. 2012; Heidari *et al*. 2012).

Eukaryotic freshwater microalgae are known mainly as a source of products such as proteins, pigments, lipids and polysaccharides (Borowitzka 2013; Koller *et al.* 2014). The limited studies that have looked at antibacterial activity put forward mainly fatty acids as the compounds

responsible; although terpenes, carbohydrates, glycoproteins, lipoproteins, bromophenols and tannins have been also suggested (Senhorinho *et al.* 2015).

Microalgae can successfully adapt to environmental stresses (Garcia-Villada *et al.* 2002). Therefore, it is probable that unique metabolites with useful activities are produced as a result (Coates *et al*. 2013). For example, extracts from an acidophilic microalga produced antibacterial activity against human pathogens (Navarro *et al*. 2016b). As a consequence, wild strains collected from extreme environments, natural or anthropogenic, represent a potential target for antibacterial screening (Challouf *et al.* 2012). However, to date these microorganisms have been subject to little investigative work for antibacterial activity.

In the Province of Ontario, Canada, there are over 250,000 lakes, and since the 19th century the province has undergone extensive mine exploration. A historic lack of mine closure plans led to abandoned sites leaving behind contaminated water bodies (Cranstone 2002). As a result, stressed aquatic environments were created, especially from acid mine drainage, which has left high metal concentrations and low pH values, conditions that lead to ecosystem disruption and microorganism selectivity (Kwiatkowski & Roff 1976; Gray 1997). Green microalgae are, however, able to adapt to extreme environments as a result of spontaneous mutation (Garcia-Balboa *et al.* 2013), or physiological acclimation through increased production of secondary metabolites (Osundeko *et al.* 2014). Therefore, eukaryotic microalgae that have adapted to extreme environments can be found in water bodies historically impacted by mining (Stokes *et al.* 1973; Eibl *et al.* 2014).

Mining-impacted water bodies are generally regarded as long-term liabilities, but we carried out the first study to determine the antibacterial potential of freshwater microalgae found

in these environments. We hypothesized that these green microalgae underwent significant adaptation, with cells producing protective secondary metabolites, including those with antibacterial activity. To this end, we investigated the diverse phylum Chlorophyta (green algae) in water bodies located within 5 km of abandoned mine sites in northern Ontario, Canada.

4.3 MATERIALS AND METHODS

4.3.1 Sampling

Collection sites near abandoned mines (Figure 4.1) were chosen based on the global positioning system coordinates provided by the Ontario Ministry of Natural Resources in the Abandoned Mines Information System (AMIS 2014). These sites were accessed by a Bell 206 helicopter, between May and September of 2014. Water samples (250 ml) were collected from the water surface in 300 ml sterile Nalgene bottles and transported to the laboratory on ice. Upon return to the laboratory, 50 ml of each sample was sent for water chemistry analysis at Glencore Process Support (formerly Xstrata Process Support), Falconbridge, Ontario, Canada, where samples were analyzed for the presence of metals through ion chromatography (IC) and pH. In the laboratory, Bristol's modified medium (Bold 1949) was added to the remaining 200 ml water samples to increase nutrients by 10%. They were then left to grow at room temperature (21 ± 2°C) for two months under 12:12 hour light:dark cycle using fluorescent light (70-80 μmol photon $m^{-2}s^{-1}$).

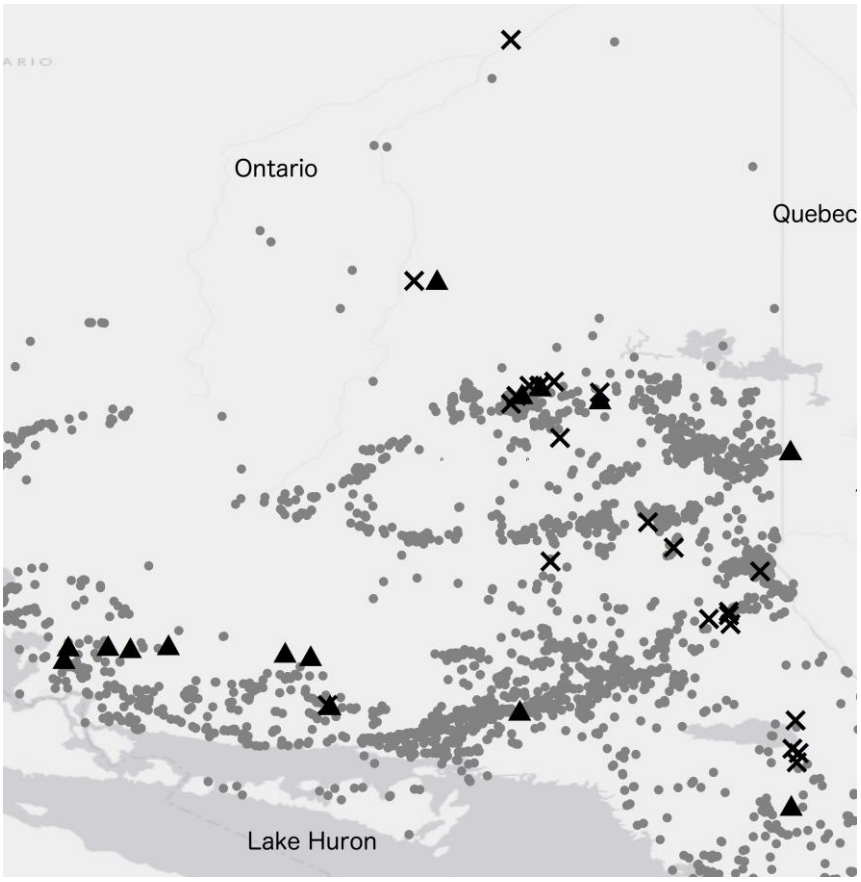

Figure 4.1. Collection sites near abandoned mines chosen based on the Abandoned Mines Information System (AMIS 2014). Water samples (250 ml) were collected from the water surface, between May and September. Abandoned mine sites in Ontario (●), water samples in which microalgae were isolated: with antibacterial activity (▲) and without antibacterial activity (✖). The map extends from latitudes 45-50°N, and longitudes78-84°W.

4.3.2 Isolation of green microalgae

One hundred water samples were randomly selected for green microalgal isolation. Once the algae visually flourished in the bottles, 20 µL from the bottom was transferred with a Pasteur pipette and streaked onto Bold's basal medium (BBM; Bold 1949) agar plates and left under 24 hours light (70-80 µmol photon $m^{-2}s^{-1}$) for 3-4 weeks until green microalgal colonies were seen.

BBM with bacteriological agar (1.5% w/v) was prepared and 40 mL poured into Petri dishes. Serial dilutions and repeated streaking of green algal colonies were performed until monocultures were obtained. Microalgal monocultures were ensured by regular observation using an inverted EVOS XL light microscope (AMG, Bothell, Washington, USA).

Morphological identification of green microalgae at generic level was performed using an inverted light microscope (AMG EVOS XL light microscope) as per Shubert (2003) and Bellinger & Sigee (2010). Cultures were tested for bacterial and fungal contamination by streaking each microalga onto nutrient agar (EMD Chemicals Inc., Darmstadt, Germany) and Sabouraud 2% glucose agar (Fluka Analytical, Seelze, Germany), and incubating over 7 days at 37°C and 25°C, respectively. Only axenic cultures were subsequently screened for antibacterial activity.

4.3.3 Extract preparation

For intracellular extract preparation, each strain was cultivated on two plates of BBM agar for 28 days, at 21 ± 2°C, under a 12:12 hour light:dark cycle (70-80 μmol photon $m^{-2}s^{-1}$). Two agar plates were used per strain to ensure sufficient biomass for screening. Extraction procedures were according to Al-Wathnani *et al.* (2012) with modifications. Cells were harvested, washed twice in sterile water, centrifuged (2000 g for 15 min), frozen at -80°C and freeze-dried. Dry biomass (108–297 mg) was weighed, and 0.08 ml of methanol mg^{-1} dry biomass was added. The tubes were agitated for 24 hr at room temperature. The procedure was repeated three times and the extracts combined. The tubes were then placed under vacuum to evaporate the solvent, after which the extracts were weighed (10–49 mg) and 100% dimethyl sulfoxide (DMSO) added to

create a final concentration of 50 mg ml^{-1}. For each strain, three extracts were prepared for screening.

4.3.4 Preliminary screening for antibacterial activity

Microalgal extracts were tested against Gram-positive *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 6051 and Gram-negative *Proteus vulgaris* ATCC 33420 and *Escherichia coli* ATCC 11303, using the agar diffusion method (Balouiri *et al.* 2016) with modifications. The bacteria were cultivated separately in nutrient broth (EMD Chemicals Inc.) for 24 h. Bacterial cultures were then adjusted to 0.5 McFarland turbidity standard and seeded onto Müller-Hinton (Oxoid, Altrincham, England) agar plates with sterile swabs. To these plates, 2 µl of each algal extract was added on top of the streaked bacterium, in triplicate, and incubated at 37°C for 18 hr. DMSO and methanol (2 µl) were used as negative controls and antibiotic discs (10 µg) of ampicillin and streptomycin (Becton, Dickinson and Company, Franklin Lakes, USA) used as positive controls.

4.3.5 Minimum Inhibitory Concentration (MIC)

For extracts exhibiting antibacterial activity against *S. aureus*, MIC was determined using a broth microdilution method (Andrews 2001). Fresh microalgal intracellular extracts were obtained from cells growing at 25°C in 300 ml of BBM in a shaker (INFORS HT Multitron Standard, Anjou, Quebec, Canada), continuously agitated at 125 rpm, under photosynthetic light (Sylvania Gro-Lux F15W / Gro T8, Padstow, New South Wales, Australia) using a 12:12 hour light:dark cycle (70-80 µmol photon $m^{-2}s^{-1}$). After 28 days, microalgae were harvested and extracted as previously described. Extracts were then filtered through a 0.2 µm sterile filter (Fisher Scientific, Ottawa, Ontario, Canada) and dissolved in 100% DMSO to 50 mg ml^{-1} stock.

S. aureus ATCC 25923 growing on Mueller-Hinton (MH) agar for 18 hr was adjusted to 0.5 McFarland turbidity standard in MH broth. Well plates were prepared with microalgal extracts (from 0.5 μg ml $^{-1}$to 1024 μg ml $^{-1}$) in a two-fold dilution in MH broth. Extracts in MH medium were inoculated into the sterile wells and the bacterium added to a final concentration of approximately 10^5 colony-forming units ml^{-1} (10^5 CFU ml^{-1}). Plates were incubated at 37°C for 18 h. Each plate contained six negative control wells (maximum 2% concentration of DMSO) in MH broth (100 μl) and was inoculated with *S. aureus* (100 μl), as well as six wells used as drug-free (inoculum-only) controls (*S. aureus* in 200 μl of MH broth). Additionally, each plate contained 12 sterility control wells with MH medium only. Ampicillin (from 0.5 μg ml $^{-1}$ to 1024 μg ml $^{-1}$) was used as a positive control. The MIC was defined as the minimum concentration of extract or ampicillin that completely inhibited *S. aureus* growth. Extracts were tested in triplicate. Figure 4.2 shows a summary of the multi-step process of microalgal screening for antibacterial activity.

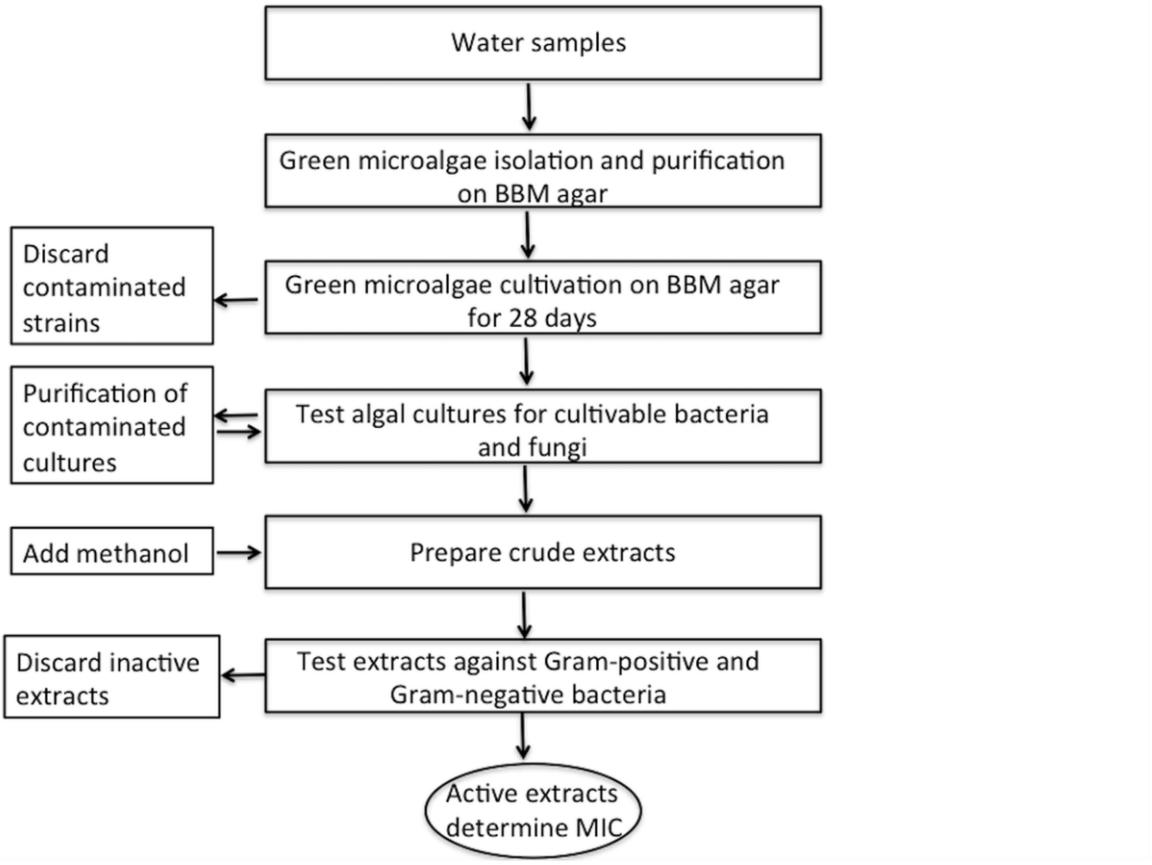

Figure 4.2. Isolation and screening procedure of freshwater green microalgae for antibacterial activity.

4.3.6 Metabolite Profiling

In order to determine the metabolic profile of the microalgal extracts showing promising antibacterial activity, they were analyzed using direct-injection liquid chromatography tandem mass spectrometry (DI-LC/MS/MS). Extracts at 50 mg ml^{-1} were prepared as previously described, frozen at -80°C and sent to The Metabolomics Innovation Centre (TMIC), University of Alberta (Edmonton, Alberta, Canada) to be analyzed.

4.3.7 Statistical Analysis

All values are reported as mean values ± standard error of the mean (SEM). Statistical analyses were performed using IBM SPSS Statistics for Macintosh, version 21 (IBM Corp., Armonk, New York USA). Metal concentrations, pH and distances from the mines between microalgae exhibiting and microalgae not exhibiting antibacterial activity were subjected to Mann-Whitney *U* test. Pearson *r* correlation coefficient tests were performed to determine association between metal concentrations and inhibition zones, between metabolite concentrations and inhibition zones as well as between each metabolite identified and pH. The point biserial correlation technique was used to determine association between metal concentrations, pH and distances from the abandoned mine sites and antibacterial activity (qualitative). Differences were considered statistically significant when $P < 0.05$.

4.4 RESULTS

One hundred water samples collected from water bodies within 5 km of abandoned mine sites in Ontario were selected for green microalgal isolation. From these, 62 samples harboured green microalgae able to grow on BBM agar, and 40 axenic strains were successfully isolated and kept on BBM agar. Each of these 40 strains came from a different water sample and was screened for antibacterial activity.

Microalgae were identified at the generic level based on cellular morphology, and six different genera were identified and isolated: *Chlamydomonas* (42.5%), *Coccomyxa* (20%), *Chlorella* (17.5%), *Scenedesmus* (10%), *Chlorococcum* (7.5%) and *Desmodesmus* (2.5%) (Table 4.1).

All strains were screened for antibacterial activity, and 15 (37.5%) showed activity against at least one of the bacterial species tested. From these positive extracts, 13 (86.7%) were from *Chlamydomonas*, one (6.7%) from *Coccomyxa* and one (6.7%) from *Scenedesmus*. Of the microalgal genera tested, 76.5% of the *Chlamydomonas* strains, 25% of *Scenedesmus* and 12.5% of *Coccomyxa* produced antibacterial activity (Table 4.1).

All microalgal extracts that showed antibacterial activity inhibited *S. aureus*. From the positive extracts, 53.3% inhibited both Gram-positive bacteria, *S. aureus* and *B. subtilis*; and 46.7% inhibited only *S. aureus* (Table 4.1). No extracts showed inhibition against the Gram-negative bacteria *E. coli* or *P. vulgaris*. Extracts obtained from the standard strain *Chlamydomonas reinhardtii* P. A. Dangeard CPCC 11 inhibited only the Gram-positive bacterium, *S. aureus*, and did not inhibit any of the Gram-negative species tested. The extract obtained from a stock culture of *Scenedesmus dimorphus* (Turpin) Kützing UTEX 1237 showed no inhibition of the species tested.

Table 4.1. Antibacterial activity (inhibition zones and MICs) of green microalgal extracts.

Extracts	Microalgal Identification	Antibacterial activity on agar plate		Inhibition zone (mm)		MIC (μg/ml)
		S.a[1]	*B.s*[2]			
M2	*Coccomyxa* sp.	+	+	5.0 ± 0	7.0 ± 0	512
M4	*Scenedesmus* sp.	+	-	5.0 ± 0	-	>1024
M6	*Chlamydomonas* sp.	+	+	7.6 ± 0.33	6.0 ± 0	128
M9		+	-	11.6 ± 0.3	-	32
M12		+	+	15 ± 0	9.3 ± 0.33	16
M13		+	+	6.3 ± 0.33	3.3 ± 0.33	>1024
M15		+	+	8.3 ± 0.33	3.6 ± 0.33	256
M16		+	-	9.3 ± 0.33	-	>1024
M17		+	-	5.0 ± 0	-	1024
M18		+	-	7.0 ± 0	-	>1024
M19		+	+	14.3 ± 0.67	8.3 ± 0.33	32
M20		+	-	11.6 ± 0.6	5.6 ± 0.3	128
M21		+	+	8.6 ± 0.3	5 ± 0	>1024
M23		+	+	11.6 ± 0.6	6.3 ± 0.3	>1024
M40		+	-	10.3± 0.3	-	32

Table 4.1. Continued

Extracts	Microalgal Identification	Antibacterial activity on agar plate		Inhibition zone (mm)		MIC (μg/ml)
		S.a[1]	*B.s*[2]			
CPCC11	*C. reihardtii*	+	-	8.3 ± 0.3	-	>1024
UTEX1237	*S. dimorphus*	-	-	-	-	NP
Ampicillin		+	+	27 ± 0	23 ± 0	2
Streptomycin		+	+	18 ± 0	17 ± 0	NP

[1] *S. aureus,* [2] *B. subtilis*, NP not performed

When extracts were screened on Mueller-Hinton agar, the diameter of the inhibition zone obtained (Figure 4.3) against *S. aureus* varied from 5 to 15 mm, with extracts M9, M12, M20, M19, M23 and M40 all showing inhibition zones above 10 mm. The inhibition zone of extracts inhibiting *B. subtilis* varied from 3 to 10 mm, with seven extracts showing an inhibition zone above 5 mm (Table 4.1).

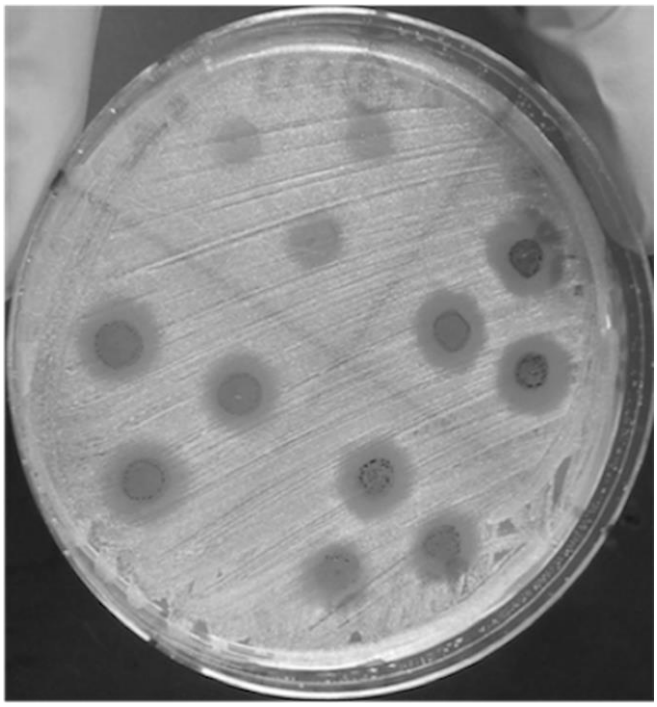

Figure 4.3. Green microalgal extracts tested against *Staphylococcus aureus*. 2 µl of each algal extract was dispensed on the bacterial lawn, in triplicate, which was then incubated at 37°C for 18 hr. UTEX 1237 extract (top quadrant) exhibiting no bacterial inhibition; M19 extract on right quadrant, M20 extract on bottom quadrant, and M12 extract on left quadrant exhibiting bacterial inhibition.

Minimum inhibitory concentration (MIC) of the extracts was then determined for the 15 showing inhibition of *S. aureus*. MIC values obtained varied from 16 to > 1024 μg ml^{-1}, with six (40%) inhibiting *S. aureus* growth at concentrations ≤128 μg ml^{-1}. From these, three extracts (M9, M19 and M40) inhibited *S. aureus* at 32 μg $ml^{-1}$and one (M12) at 16 μg ml^{-1}. However, extracts from the control, *C. reinhardtii* CPCC 11, inhibited *S. aureus* only at a concentration > 1024 μg ml^{-1} (Table 4.1).

Chemical analyses were performed to determine the chemistry of the water samples, and concentrations of Fe, Ni, Cu, Zn, As, Cd and Pb were calculated, as well as pH (Table S1). Metal concentrations and pH of the water samples, as well as the distances from the abandoned mines, were compared for microalgae exhibiting and not exhibiting antibacterial activity, and no significant differences were observed (Table 4.2).

Table 4.2. Mean values of metal ions, pH, and sum of metal ions from water sample analysis and distance of water sample from abandoned mine sites.

Characteristic	Positive microalgae for antibacterial activity (*n* =15)	Negative microalgae for antibacterial activity (*n* =25)
Fe (ppm)	2.2 ± 1.5	0.4 ± 0.2
Ni (ppm)	0.07 ± 0.05	0.01 ± 0.01
Cu (ppm)	0.06 ± 0.05	0.06 ± 0.03
Zn (ppm)	3 ± 2.8	0.7 ± 0.6
As (ppm)	0.001 ± 0.0001	0.009 ± 0.008
Cd (ppm)	0.045 ± 0.04	0.004 ± 0.002
Pb (ppm)	0.005 ± 0.003	0.07 ± 0.07
pH	6.3 ± 0.4	6.8 ± 0.2
Sum of metal concentration	5.4	1.3
Distance from the abandoned mine sites (km)	3.13 ± 0.44	2.9 ± 0.28

Results expressed as mean values ± standard error. Statistical analysis based on Mann-Whitney *U* test.

To investigate the relationships between the metals analyzed, pH, distance from the mines, and microalgal antibacterial activity, correlation tests were performed. The analyses showed no significant correlations (Table 4.3).

To partially identify metabolites in the methanolic extracts exhibiting the most promising antibacterial activity against *S. aureus*, extracts were analyzed using DI-LC/MS/MS. A wide panel of metabolites was identified, including amino acids, glycerophospholipids, sphingolipids, acylcarnitines and biogenic amines (Table S2).

To ascertain whether there was a relationship between the metabolites analyzed and the antibacterial activity obtained in this study, correlation tests were performed and the analysis showed no significance (Table 4.3).

Table 4.3. Correlation (*r*) between water sample measures and antibacterial activity, between distance from the mines and antibacterial activity, between metabolites and pH, and between metabolites and antibacterial activity.

Measures	*r*	*P*
Sum of metal concentration per sample vs antibacterial activity	- 0.1	0.19
pH vs antibacterial activity	0.2	0.2
Distance from the mine sites vs antibacterial activity	- 0.07	0.6
Sum of metabolite concentrations per extract vs inhibition zones	0.24	0.6
Sum of amino acid concentrations vs pH	0.25	0.5
Sum of glycerophospholipids vs pH	- 0.02	0.9
Sum of sphingolipids vs pH	0.23	0.6
Sum of acylcarnitines vs pH	0.18	0.7
Sum of biogenic amines vs pH	0.34	0.4
Sum of amino acids vs inhibition zones	0.19	0.67
Sum of glycerophospholipids vs inhibition zones	0.56	0.19
Sum of sphingolipids vs inhibition zones	0.52	0.2
Sum of acylcarnitines vs sum of inhibition zones	- 0.54	0.2
Sum of biogenic amines vs sum of inhibition zones	0.04	0.9

4.5 DISCUSSION

Microalgae under stressing conditions secrete substances that are not regularly produced (Fogg 2001), and these metabolites may function as anti-predator and anti-infection and they may also aid in communication (Coates *et al.* 2013). The current study is, to our knowledge, the first to verify that freshwater green microalgae from environments in proximity to abandoned mine sites can produce compounds that inhibit bacterial growth. The results showed not only that these microalgae exhibit antibacterial activity against Gram-positive bacteria, but also that these environments harbour a higher percentage of antibacterial producers (37.5%) than indicated by previous studies on microalgal screenings. For example, screening of freshwater algae (including green microalgae and blue-green algae) carried out by Cannell *et al.* (1988) found only 12% positive strains. Similar screenings of marine microalgae by Kellam & Walker (1989) and Mudimu *et al.* (2014), found that 21% and 29%, respectively, of algal extracts exhibited antibacterial activity.

Another important finding was the promising antibacterial activity that certain microalgal extracts, M6, M9, M12, M19, M20 and M40, exhibited against *S. aureus*. The active concentrations were very low (16-128 μg ml $^{-1}$), notably lower than previously reported values (Table 4.1). Extracts of the green alga, *Cosmarium* sp., collected from a hot spring, showed *S. aureus* inhibition at 150 μg ml $^{-1}$ (Challouf *et al.* 2012), and extracts from *Chlorococcum humicola* (Nageli) Rabenhorst inhibited different bacterial species from 10,000 to 22,000 μg ml $^{-1}$ (Bhagavathy *et al*. 2011). Considering that the crude extracts used in this work were a mixture of many compounds and that microorganisms usually produce active compounds at very low

concentrations (Borowitzka 1995), purification would be expected to significantly further decrease the MIC (Ordog *et al.* 2004).

Our microalgal screening established that all methanolic extracts showing antibacterial activity inhibited Gram-positive bacteria; although no inhibition was seen against Gram-negative bacteria. Debro & Ward (1979) and Cannell *et al.* (1988) obtained similar results from freshwater algal extracts. Gram-negative bacteria are less susceptible to antibacterial compounds, because unlike Gram-positive bacteria, they have a protective outer membrane (Silhavy *et al.* 2010). On the other hand, Navarro *et al*. (2016b) observed antibacterial activity in extracts from the green microalga *Coccomyxa onubensis* strain SAG2510 (Fuentes *et al* 2016*)* mostly against Gram-negative bacteria. According to their study, no promising antibacterial activity was detected from methanolic extracts. This difference in results may be explained by the different species/strains, since in our study no activity against Gram-negative bacteria was observed from any of the extracts analyzed.

Of the Gram-positive bacteria we tested, *S.aureus* was more susceptible to the extracts than *B. subtilis.* Kellam & Walker (1989) obtained similar results while screening marine green microalgae. However, Cannell *et al.* (1988) found *B. subtilis* to be far more susceptible than *S. aureus* to freshwater algal extracts (including those from unicellular and filamentous eukaryotic algae, as well as cyanobacteria). The active compounds were not identified, and the bacterial mode of action was unknown. Hence the ability of these extracts to inhibit one Gram-positive species better than the other has not been explained. However, the results from the current study are very encouraging as *S. aureus* is an opportunistic species (Pantosti *et al.* 2007) that has exhibited a multi-drug resistant phenotype, including resistance to last-resort antibiotics (Wilson *et al.* 2003; Zhanel *et al.* 2008; Tarai *et al*. 2013; Cavalcante *et al.* 2014).

No significant correlations between antibacterial activity of microalgae and metals analyzed, pH or distance from the mine sites were obtained. However, based on the number of samples harbouring microalgae for antibacterial activity and the promising ability of these microalgae to inhibit *S. aureus*, environments near abandoned mine sites seem to have a higher than average potential for harbouring antibacterial producers, which warrants further investigation.

It is possible that factors associated with abandoned mine sites, other than the isolated metals or pH, could influence the production of antibiotics by the green microalgae tested. One possible factor is the presence of other organisms, as studies have shown that microalgae produce antibacterial compounds when present in a competitive environment (Lustigman 1988; Kokou *et* al. 2012). Although fewer taxa are present in contaminated areas near abandoned mine sites than in unpolluted areas, the same phyla are found in both environments, with some organisms more prevalent in contaminated areas (Say & Whitton 1981).

An interesting finding is that among the antibacterial microalgae obtained, *Coccomyxa* sp. (extract M2) and *Chlamydomonas* sp. (extract M23) came from water samples exhibiting extremely low pH (both 2.9; Table S1). Interestingly, both extracts demonstrated antibacterial activity against *S. aureus*. Navarro *et al.* (2016b) also observed that a strain of *C. onubensis* obtained from an acidic site (pH 2.5) exhibited antibacterial activity. These results suggest that green microalgae from extremely acidic water could be associated with the production of compounds with antibacterial activity and that future studies should target these extreme environments. Microalgae growing in highly acidic conditions demonstrate their adaptive nature, and significant changes in these organisms, such as increased internal buffer capacity, low

conductance of the plasma membrane and/or an increased ability to export H^+ are not surprising (Gimmler 2001).

Another factor that could influence microalgal antibacterial activity is the variation in temperature to which these microorganisms are exposed in fresh water in Canada. Yearly surface temperature of lakes in Ontario usually varies from 0°C to approximately 24°C (Schindler *et al*. 1996), with most being ice-covered between December and March (Canadian Ice Service publications 2010). Variations in temperature affect permeability, metabolic regulation and rate of intracellular reactions in plants, with effects on growth and secondary metabolism (Ramakrishna & Ravishankar 2011). Also, variation in temperature has been shown to influence the ability of algae to accumulate secondary metabolites with antibacterial activity (Amade & Lemee 1998).

Our analysis of metabolite concentration detected in the most active extracts of green microalgae showed no correlation with the antibacterial activity seen in this study. These results suggest that the metabolites that we identified do not have a direct association to the antibacterial activity in these microorganisms. Yet, it is possible that some of the metabolites identified are indirectly related to the production of antibacterial activity of freshwater green microalgae. Since the content of methanolic extracts from green microalgae exhibiting antibacterial activity is still largely unknown, the identification of metabolites present in these extracts warrants further study.

The results of this study indicate that freshwater bodies near abandoned mine sites have the potential to harbour green microalgae that exhibit promising antibacterial activity against *S. aureus*. As these polluted water bodies are usually regarded as having no value, indeed often as problematic, considering their potential biochemical use could turn them from liabilities into

assets. However, since no clear association was found between antibacterial activity and metals analyzed or pH, future studies should explore this relationship to determine environmental thresholds that cause these microalgae to exhibit antibacterial activity.

4.6 ACKNOWLEDGMENTS

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CHAPTER 5: THE INFLUENCE OF HARVESTING TIME ON THE ANTIBACTERIAL ACTIVITY OF GREEN MICROALGAL EXTRACTS AND THEIR EFFECT ON VIABILITY OF NON-MALIGNANT AND MALIGNANT CELL VIABILITY

(Original Research)

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[SUBMITTED TO PHYCOLOGICAL RESEARCH]

5.1 ABSTRACT

Microalgae are being investigated for their ability to produce metabolites that have commercial value, including those with antibacterial activity. However, little is known about at what point during the microalgal growth phase they produce the maximum amount of metabolites exhibiting antibacterial activity. Furthermore, as research on antibacterial activity progresses, the effect of microalgal extracts on mammalian cells should be assessed.

In this study, extracts from *Chlamydomonas* sp. that have previously shown activity against *Staphylococcus aureus* were investigated for antibacterial activity throughout their growth phase. Moreover, the effect of these microalgal extracts on the viability of non-malignant (MCF-10A and 184B5 cells) and malignant human cell lines (A2780 and MCF-7) was examined. The results demonstrated that *Chlamydomonas* sp. extracts showed higher antibacterial activity when harvested towards the end of the exponential phase. In addition, the extracts reduced cell viability of malignant cells at specific concentrations. The viability of non-malignant cells was not reduced by the extracts, but instead there appeared to be an increase in viability of these non-malignant cell lines upon exposure to the extracts.

Keywords: Antibiotics, Cancer, Eukaryotic microalgae, MTT, Viability

5.2 INTRODUCTION

Microalgae have been investigated for their production of compounds with commercial interest, such as polysaccharides, proteins, vitamins, fatty acids and pigments (Cardozo *et al* 2007; Sasso *et al.* 2012; Skjånes *et al.* 2012; Abd El Baky & El-Baroty 2013; Koller *et al* 2014; Michalak & Chojnacka, 2015). These compounds are either the result of microalgal primary metabolism, or the less understood secondary metabolism (Trick *et al.* 1984), and can provide important biological activities. These include antioxidant, antitumoral, antialgal, antiviral, and antifungal activities (Mudimu *et al.* 2014; Ruffell *et al.* 2016; Pina-Perez *et al* 2017).

Investigations with microalgal extracts have revealed that certain organisms can produce metabolites that inhibit pathogenic bacteria (Navarro *et al.* 2016b; Senhorinho *et al*. 2018). Antimicrobial activity from natural sources is usually associated with the production of secondary metabolites (Leflaive & Ten-Hage 2007), those are produced as a result of the accumulation of intermediate and end products from the organism's primary metabolism (Malik 1980). Their production is thought to usually occur towards the end of the exponential phase or during the stationary phase of growth (Skulberg 2000; Leflaive & Ten-Hage 2007).

For economic reasons if microalgae are to be screened for antibacterial activity, the harvesting time should be ideally at the earliest time of production in the growth curve and/or when the organisms produce the maximum amount of bioactive compounds. However, since the objective of screening is to quickly cover large numbers of organisms to select promising strains for further study, testing the antibacterial activity of each microalga over the entire growth curve would be laborious and unreasonable. Therefore, screenings are usually conducted after choosing

a harvesting day in either the exponential or stationary phase of growth (Najdenski *et al.* 2013; Li *et al.* 2016).

Harvesting time has, however, been suggested as important to antibacterial activity of *Scenedesmus* strains (Aremu *et al.* 2014). Although the number of studies on antibacterial activity of microalgae has been increasing (Mudimu *et al.* 2014; Lauritano *et al.* 2016, Corona *et al.* 2017; Senhorinho *et al.* 2018), little is known about the period in the growth curve at which microalgae start to produce antibacterial activity. Knowing when antibacterial activity is likely to occur would help researchers speed up the overall screening process by harvesting microalgae at the earliest point in the growth curve.

In antibiotics discovery, once antibacterial activity is observed, determining the effect of the extract on mammalian cells is an important next step. That is, a promising new antibiotic needs to show low or no toxicity towards the mammalian cells (Chopra 2013). Microalgal extracts from eukaryotic and prokaryotic microalgae have been shown to decrease viability of both healthy and malignant mammalian cells (Bechelli *et al.* 2010; Hernandez *et al* 2016). Extracts from the green microalga *Chloromonas* sp. showed an anti-proliferative effect towards malignant cells, whilst no such effect was observed on non-malignant cells (Suh *et al.* 2017).

Interestingly, studies have shown that microalgal extracts can contain compounds with growth inhibitory activity towards malignant cells and have been investigated as potential anticancer therapies (Hong & Luesch 2012). Microalgal extracts exhibiting antibacterial activity seem to also have an inhibitory effect on viability of malignant cells (Ordog *et al.* 2004). Since natural sources, such as microorganisms, can produce important compounds with anticancer activity, the effects of green microalgal extracts with promising antibacterial activity on

mammalian cell viability should be also evaluated. Once the effect on mammalian cell viability has been determined, it would be also interesting to determine if there is a difference between the response by malignant and non-malignant cells.

To address these issues, we identified five *Chlamydomonas* sp., from screening freshwater green microalgae in water bodies near abandoned mine sites in Ontario, Canada, that showed promising antibacterial activity against *Staphylococcus aureus*. The purpose of this study was to determine the earliest period of growth in which freshwater green microalgal extracts demonstrate antibacterial activity against *S. aureus*, and to also provide an initial assessment as to *in vitro* impact of the extracts on viability of non-malignant and malignant human cell lines.

5.3 MATERIALS AND METHODS

5.3.1 Microalgal strains and growth conditions

Five wild *Chlamydomonas* sp. (M6, M9, M12, M19, M40) collected from freshwater lakes near abandoned mine sites in Ontario, Canada, where microalgae survive at high metal levels and low pH, were used in this study (Senhorinho *et al.* 2018). The initial screening was on day 28 (exponential phase) of microalgal growth, and the extracts from the five microalgae showed promising antibacterial activity against Gram-positive bacteria, particularly the human opportunistic pathogen *Staphylococcus aureus* ATCC 25923 (Senhorinho *et al.* 2018). Culture collection strains, *Chlamydomonas reihardtii* CPCC11 (Cr), which previously showed antibacterial activity on day 28 of growth, and *Scenedesmus dimorphus UTEX* 1237 (Sd), which did not show any antibacterial activity after growing for 28 days were also included in this work.

Isolates of green microalgae grown for 10 days (10 to 22 mg dried biomass) on Bold's basal medium (BBM) agar plates (at 21±2°C, under a 12:12 hour light:dark cycle, 70-80 μmol photon $m^{-2}s^{-1}$) were transferred to flasks containing 500 ml of liquid BBM. They were then grown for a further 49 days in an incubator (INFORS HT Multitron Standard, Anjou, Quebec, Canada), at 25°C, continuously agitated at 125 rpm, and under photosynthetic light (Sylvania Gro-Lux F15W / Gro T8, Padstow, New South Wales, Australia) using a 12:12 hour light:dark cycle (70-80 μmol photon $m^{-2}s^{-1}$). Axenic cultures were used in this study.

5.3.2 Microalgal growth

During growth, one milliliter samples were analyzed in triplicate every three days for 48 days using a spectrophotometer (UV-1700 UVVIS, Shimadzu, Tokyo, Japan) at a wavelength of 550 nm.

5.3.3 Microalgal extract

Each microalga was harvested every seven days over 49 days, washed twice with sterile water, centrifuged at 2000 xg for 15 min, frozen at -80°C, freeze-dried and weighed. The resultant biomass was immediately used for preparation of extracts. Extracts were obtained following the methodology described by Al-Wathnani *et al.* (2012) with modifications.

To the biomass obtained, 0.08 ml of methanol per mg of dry biomass was added and agitated for 24 hr at room temperature. The biomass was centrifuged at 2000 xg for 15 min and the supernatant removed. This procedure was repeated three times, the extracts combined and filtered through a 0.2 μm sterile filter (Nalgene, Fisher Scientific, Ottawa, Ontario, Canada). Tubes with extracts were then placed under vacuum for solvent evaporation after which, extracts

were weighed and 100% dimethyl sulfoxide (DMSO) added to create a final concentration of 50 mg/ml.

Microalgal extracts to test on mammalian cells were prepared as described above. In order to compare the concentrations of extracts affecting mammalian cells with the concentrations affecting *S. aureus* cells, extracts were prepared with microalgal cells harvested on day 28, since the minimum inhibitory activity (MIC) of the extracts analyzed in this study was previously tested on day 28, exhibiting promising results against *S. aureus* ATCC 25923 (Senhorinho *et al.* 2018).

5.3.4 Antibacterial activity test

Antibacterial activity tests were performed using the agar diffusion method according to Balouiri *et al.* 2016 with modifications. The extracts were tested against the Gram-positive strain *Staphylococcus aureus* (ATCC 25923). This bacterium was grown in nutrient broth (EMD Chemicals Inc., Darmstadt, Germany) for 24 hr, adjusted to 0.5 McFarland turbidity standard and seeded onto Müller-Hinton (Oxoid, England, UK) agar plates using sterile swabs. Two microliters of each extract was added on top of the bacterial lawn, in triplicate and incubated at 37°C for 18 hr. DMSO and methanol (2 μL) were used as negative controls and antibiotic discs (10 μg) of ampicillin and streptomycin (Becton, Dickinson and Company, Franklin Lakes, USA) used as positive controls. Experiments were performed as three biological replicates in triplicate.

5.3.5 Mammalian cell culture conditions

Two malignant cell lines, MCF-7 (breast carcinoma) and A2780 (human ovarian carcinoma); and two non-malignant cell lines, MCF-10A and 184B5 (non-tumorigenic mammary

epithelial lines) were used in this study. A2780 was obtained from the European Collection of Cell Culture and maintained in RPMI-1640 (Hyclone Laboratories, Logan, UT, USA) culture medium containing 10% fetal bovine serum (FBS). MCF-7 cells, from the American Tissue Culture Collection (ATCC), were maintained in MEM/EBSS (minimum essential medium with Earle's salt solution: Gibco, Waltham, MA, USA) culture medium supplemented with 10% FBS and 10 μg/ml of bovine insulin. MCF-10A (supplied by Dr. C. Lanner, Northern Ontario School of Medicine) was maintained in DMEM/F12 (Dulbecco's modified eagle medium nutrient mixture F12: Gibco, Waltham, MA, USA) supplemented with 5% equine serum, 10 μg/ml of insulin, 20 ng/ml of epidermal growth factor (EGF), 100 ng/ml of cholera enterotoxin and 0.5 μg/ml of hydrocortisone. Cell culture of 184B5 (ATCC CRL-8799) (supplied by Dr. C. Lanner, Northern Ontario School of Medicine) was maintained in MEGM (mammary epithelial cell growth medium: Clonetics, San Diego, CA, USA) with 1 ng/ml of cholera enterotoxin added. All cells were maintained in a humidified incubator at 37°C with 5% CO_2. No antibiotics were added to the cell cultures.

5.3.6 Mammalian cell treatment and viability test

Mammalian cell lines were grown in Corning T75 tissue culture flasks until cells reached confluence. Cells were then seeded (10^4 cells/well) onto flat-bottomed 96 well plates for 24 h, and each well was treated with microalgal extract at various concentrations (5, 10, 25, 50, 100 and 150 μg/ml) for 24 h. Extracts for the cell treatments were obtained as previously described, after microalgal growth for 28 days. DMSO was present in the cultures at a maximum of 0.3%. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to Khurana *et al.* (2014). Once the incubation time was

completed, MTT dye was added at 1/10th volume (5 mg/ml in PBS) (Sigma-Aldrich, Oakville, Ontario, Canada). Cells were then incubated at 37°C with 5% CO_2 for 4 hours, after which the medium was aspirated and 50 µl of DMSO added. The plates were then incubated in the dark for 15 min.

The absorbance at 570 nm was measured on a plate reader (Bio Tek, PowerWave XS: Winooski, Vermont, USA). A cell control (untreated cells), DMSO control (0.01% - 0.3%) and blank (medium with no cells plus each extract at each concentration used plus MTT, and plus DMSO) were added in triplicate. Three independent experiments were carried out in triplicate. Optical densities of samples and vehicle were normalized to controls. The readings of the cell control, with untreated cells, were considered as 100% survival. The percentage survival of the mammalian cells was calculated according to van Meerloo *et al.* (2011) as follows:

$$\% \text{ of viable cells} = (abs_{samples/vehicle} - abs_{blank} / abs_{control} - abs_{blank}) \times 100$$

5.3.7 Statistical analysis

All values are reported as mean values ± standard error of the mean (SEM). Statistical analyses were performed using IBM SPSS Statistics for Macintosh, version 21 (IBM Corp., Armonk, N.Y., USA). Repeated measures analysis of variance (ANOVA) with a Greenhouse-Geisser correction followed by Bonferroni's test was used to discriminate differences in antibacterial activity from *Chlamydomonas* sp. extracts between the different harvesting times. T-tests were performed, to discriminate significant differences between microalgal extracts and controls when mammalian cells were treated. T tests with the Welch-Satterthwaite method were used when the factors failed the assumption of equal variance. Differences were considered statistically significant when $P < 0.05$.

5.4 RESULTS

5.4.1 Microalgal growth. Microalgal biomass was first harvested on day seven, which was the beginning of the exponential phase, and last harvested on day 49, which was the early stationary phase (Figure 5.1). Microalgal growth was analyzed with spectrophotometry over the entire period (Figure 5.2).

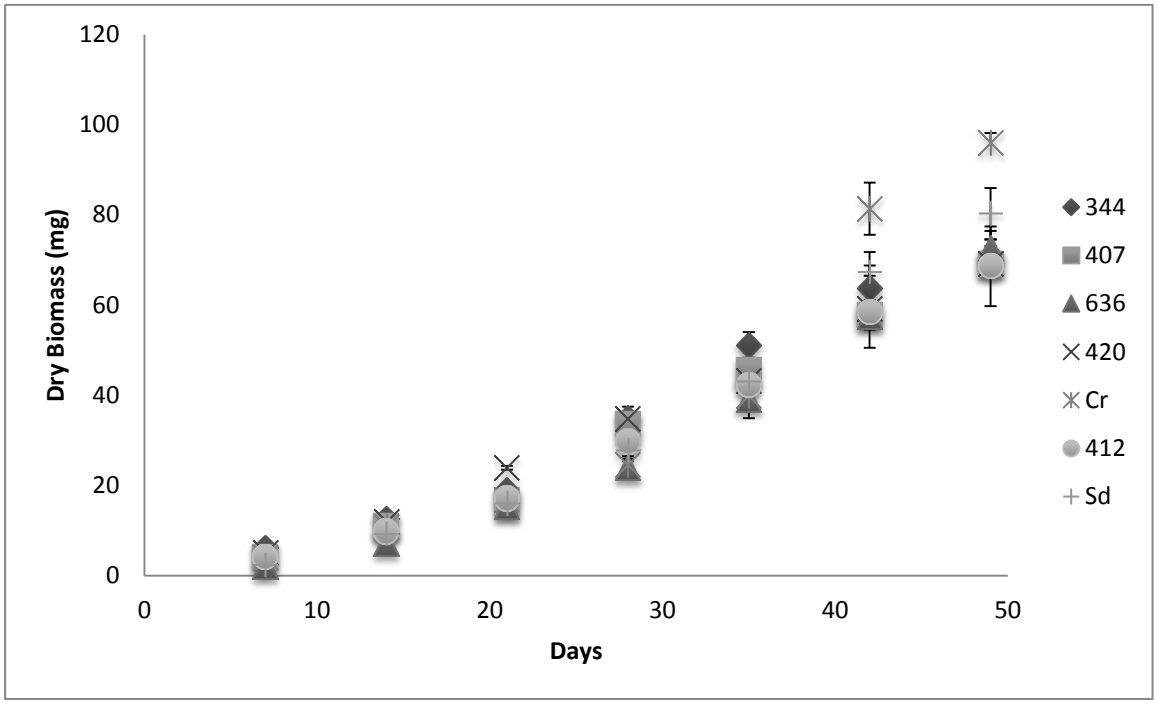

Figure 5.1. Microalgal biomasses harvested every seven days for 49 days. Microalgal cells in liquid BBM were harvested during cell growth, washed with water, centrifuged, frozen at -80°C, freeze-dried and weighted. The resultant biomasses are expressed as mean ± SEM (n = 3). Cr, extract from *C. reinhardtii* CPCC11; Sd, extract from *S. dimorphus UTEX* 1237.

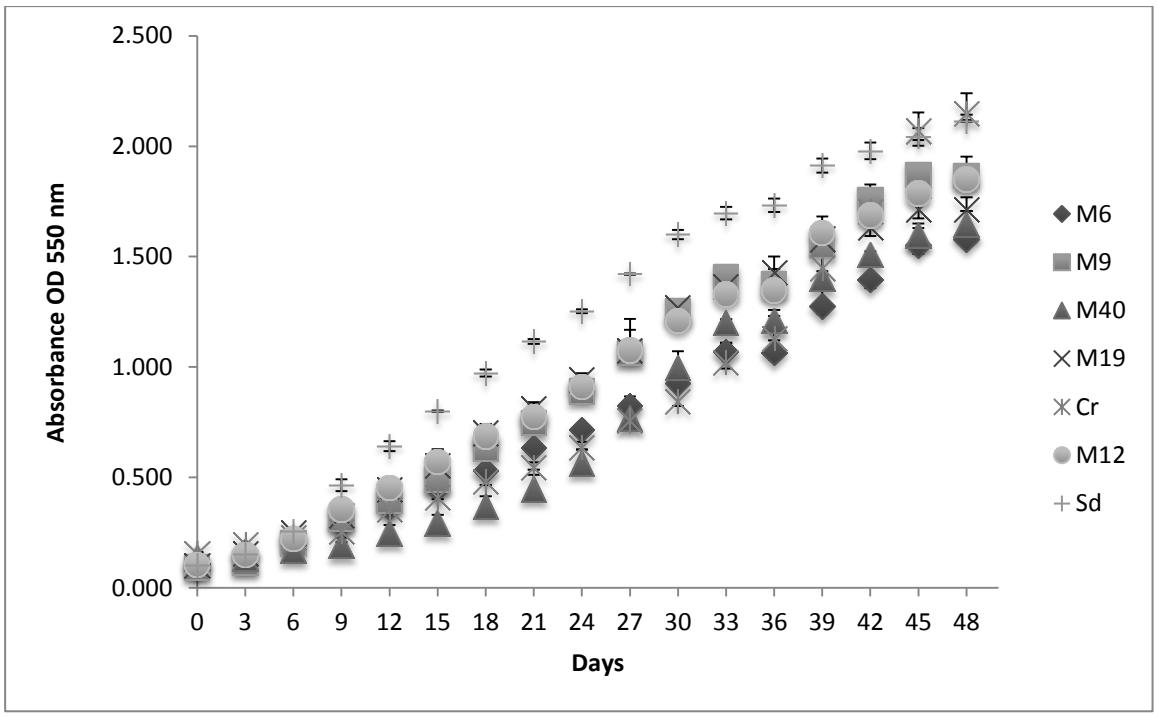

Figure 5.2. Microalgal growth rate analyzed every three days for 48 days. Microalgal cells in liquid BBM were harvested during cell growth and analyzed through spectrophotometry at 550 nm. Results are expressed as mean ± SEM (n = 3). Cr, extract from *C. reinhardtii* CPCC11; Sd, extract from *S. dimorphus UTEX* 123.

5.4.2 Antibacterial activity. Extracts of all *Chlamydomonas* sp. inhibited *S. aureus*. On the other hand, extracts from the culture collection strains, *C. reinhardtii* and *S. dimorphus*, showed antibacterial activity until day 28 and 14, respectively. The controls, DMSO and methanol, did not show any inhibition of *S. aureus* growth (data not shown). However, a significant difference in antibacterial activity was observed among *Chlamydomonas* sp. harvesting times ($P < 0.05$). Extracts obtained from *Chlamydomonas* sp. showed significantly ($P = 0.014$) higher bacterial inhibition when harvested towards the end (day 35) of the exponential phase, and lower antibacterial activity at the early (day 49) stationary growth phase (Table 1).

Table 5.1. Antibacterial activity of green microalgal extracts collected at different times during microalgal growth against *S. aureus.*

Extracts	Day 7 [*a]	Day 14[*a]	Day 21	Day 28[*a]	Day 35 [*b]	Day 42	Day 49 [*a]	MIC[3] (μg/ml)
	Zone in mm	Zone in mm	Zone in mm	Zone in mm	Zone in mm	Zone in mm	Zone in mm	on day 28 (Senhorinho *et al.* 2018)
M6	11.5± 0.5	10.3± 0	0	11.6± 0.5	15.8± 0.1	13.6± 0.6	7.2± 0.1	128
M9	13 ± 0.7	11.1± 0.1	9.8± 0.4	11.5± 1.2	15.6± 0.6	13.8± 0.3	5.7± 0.2	32
M12	10.9± 0.7	10.8 ± 0.1	8 ± 0	10.8± 1.6	15.4± 0.3	15.7± 0.8	8.3± 0.2	16
M19	12.7 ± 0.3	9.1 ± 0.1	9.6± 0.3	11.8± 1	15.8± 0.4	14.3± 0.3	6.5± 0.5	32
M40	10.3± 1	10 ± 1.3	0	8.4± 1	13.3± 0.2	0	0	32
Cr[1]	12.1± 1.7	11.4± 0.4	8.2± 0	7.2± 0.2	0	0	0	>1024
Sd[2]	12.8 ± 1.2	9 ± 0	0	0	0	0	0	Not tested

Results are from measuring the zones of inhibition around extracts. [*]Significant when zones of inhibition from extracts of *Chlamydomonas* sp. were analyzed. Different letters indicate significant difference based on repeated measures ANOVA ($P < 0.05$). Controls: ampicillin (27 mm ± 0); streptomycin (18 mm ± 0). Results are expressed as mean values ± SEM ($n = 3$).[1] Cr, extract from *C. reinhardtii* CPCC11; [2] Sd, extract from *S. dimorphus UTEX* 1237; [3]MIC (the minimum concentration of extracts that completely inhibited *S. aureus* growth).

5.4.3 Effect of microalgal extracts on mammalian cell viability. Non-malignant and malignant cells were incubated for 24 hours with each microalgal extract at various concentrations (5 – 150 μg/ml). The two non-malignant cell lines, MCF10A and 184B5, had no decrease in cell viability when compared to the control, but instead an increase in viability at some extract concentrations were observed, particularly for the cell line 184B5 (Figure 5.3). On the other hand, at some concentrations of extracts, the malignant cells, MCF-7 and A2780, showed a significant decrease in cell viability when compared to the control (untreated cells) (Figure 5.3).

The 184B5 cell line showed a significant increase in cell viability when cells were treated with microalgal extracts at most concentrations tested, particularly extracts M40 and Sd (Figure 5.3). The MCF10A cell line also exhibited increased viability when treated with some concentrations of microalgal extracts, in particular extracts from M6 and Cr (Figure 5.3). Moreover, MCF10A cells showed a significant increase in viability when treated with some concentrations of extracts from M9 and Sd (Figures 5.3C, 5.3D, 5.3E), with 150 μg/ml of M19 extract (Figure 5.3G) and 5 μg/ml of M40 extract (Figure 5.3B).

Of the two malignant cell lines, A2780 line was the most significantly affected by all microalgal extracts in terms of cell viability, but particularly by extracts from M9, M40 and M12 (Figure 5.3). Extract from M9 exhibited the strongest effect on A2780 cell viability in a concentration-dependent manner, reducing 73% of viability when cells were treated with the highest concentration tested of 150 μg/ml (Figure 5.3G). Extract from M40 exhibited 32% decrease in cell viability at the low concentration of 10 μg/ml (Figure 5.3C) and 54% decrease in viability at the maximum concentration tested (Figure 5.3G). Moreover, extract from M12

reduced A2780 viability at 50 μg/ml, 100 μg/ml and 150 μg/ml, with a reduction of 65% of viability at the highest concentration (Figure 5.3G).

A less pronounced effect was seen on A2780 cell viability when cells were treated with extract M6, with a maximum of 31% reduction in cell viability at the highest concentration tested (Figure 5.3G). Furthermore, the extracts from the culture collections, Cr and Sd, had a significant impact on A2780 cell viability at all concentrations tested, with respective decreases of 49% and 56%. Extract from M19 exhibited the least negative effect on A2780, with only significant decreases in cell viability at concentrations of 10 μg/ml (24%, Figure 5.3C) and 25 μg/ml (30%, Figure 5.3D). There was no significant difference in viability with the control at the higher concentrations tested (Figures 5.3E, 5.3F, 5.3G).

MCF7 cells were negatively affected by extracts from M9 and M12 at only the highest concentrations tested (100 μg/ml and 150 μg/ml, Figures 5.3F, 5.3G). The cell viability was reduced by 59% and 39% when treated with 150 μg/ml of extracts from M9 and M12, respectively (Figure 5.3G). MCF7 cells also exhibited a significant increase in cell viability when treated with 5 μg/ml to 50 μg/ml of extract from M40 (Figures 5.3B, 5.3C, 5.3D, 5.3E), 100 μg/ml and 150 μg/ml of extract from M6 and 50 μg/ml of extract from Cr. No effects were observed with MCF7 cell viability when the cells were treated with extracts from M19 and Sd (Figure 5.3).

At the concentrations where microalgal extracts inhibited *S. aureus* growth (16 – 128 μg/ml) (Table 1), non-malignant mammalian cells showed no decrease in cell viability. Of the malignant cells tested, A2780 cells were negatively affected from 5 μg/ml and MCF7 cells affected by concentrations above 100 μg/ml.

The microalgal extract that exhibited the highest antibacterial effect (Table 1) against *S. aureus*, from M12, did not decrease non-malignant cells viability. However, a significant decrease in MCF7 and A2780 viability was observed when cells were treated with the highest concentrations of this extract (Figures 5.3E, 5.3F, 5.3G).

The use of DMSO from 0.01% to 0.3% in the cell cultures did not significantly interfere with the cell viability of any of the cell lines tested in this study (Figure 5.3A).

Figure 5.3. Effect of microalgal extracts on non-malignant and malignant cells. One asterisk indicates significant differences to the control (control = cells only). Cr, extract from *C. reinhardtii*; Sd, extract from *S. dimorphus.*

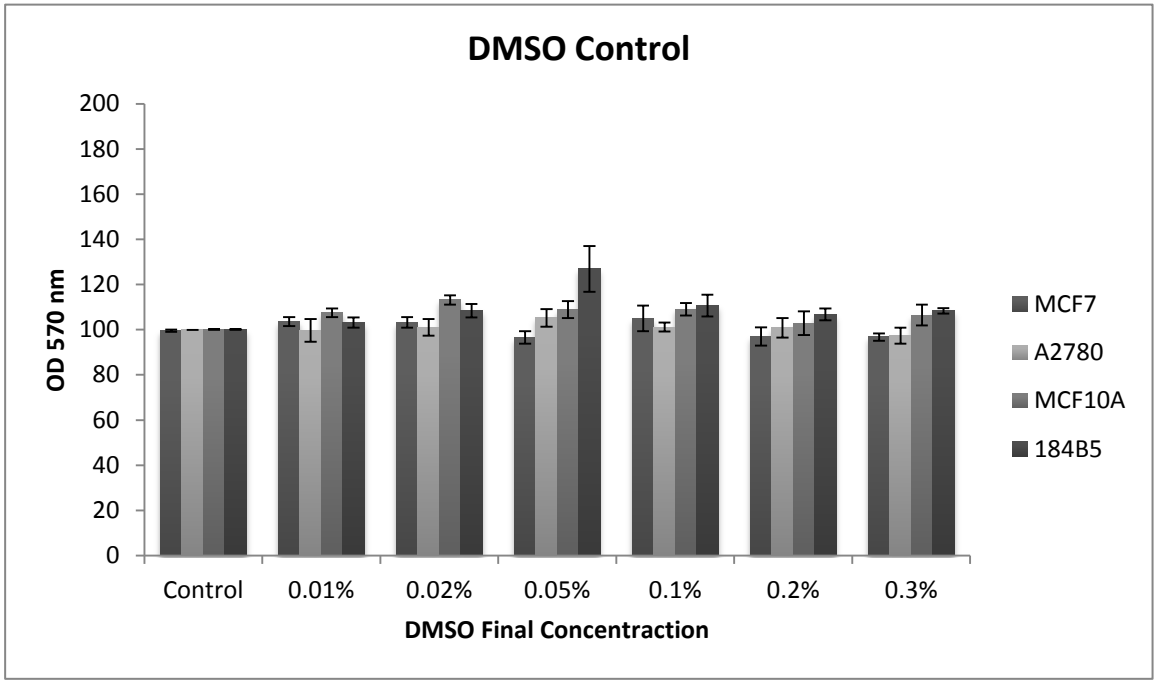

Figure 5.3A.Effect of DMSO on mammalian cell viability. Cells were treated with the same concentrations of DMSO as found in the treatments of mammalian cells. Cells were exposed to different concentrations of DMSO for 24 hours and MTT assay was performed (*n*= 3).

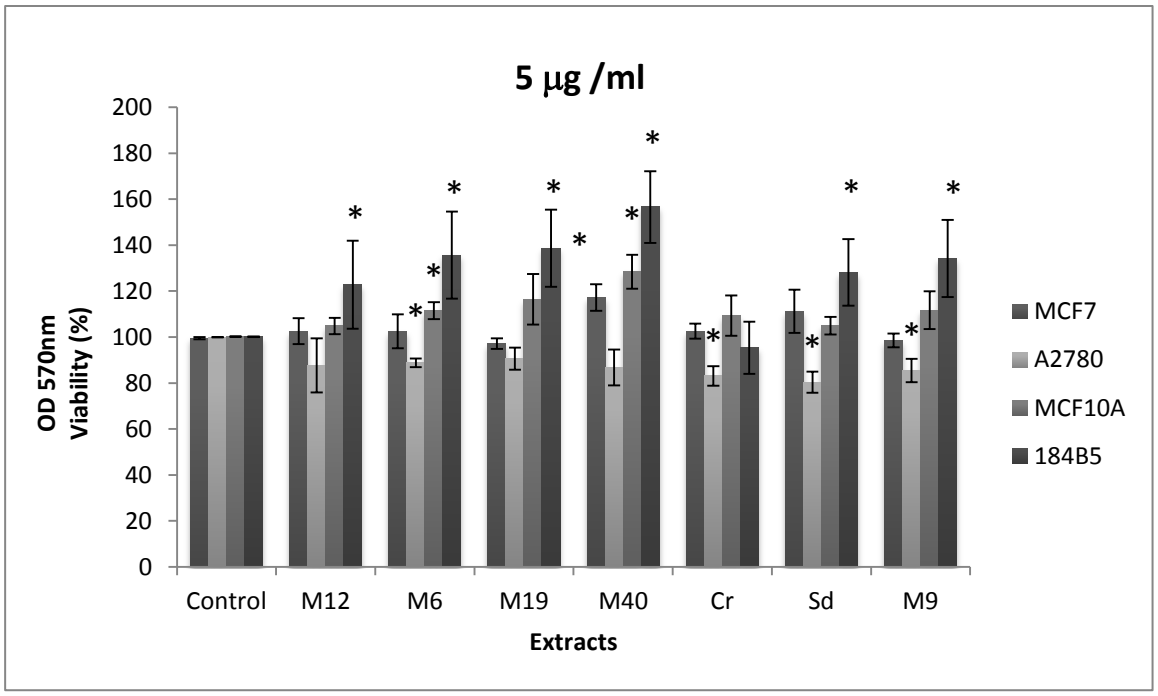

Figure 5.3B. Effect of 5 μg/ml of microalgal extracts on mammalian cell viability. All mammalian cells were exposed to 5 μg/ml of microalgal extracts and incubated for 24 hours. The cell viability was assessed by MTT assay ($^{*}P < 0.05$, T-test).

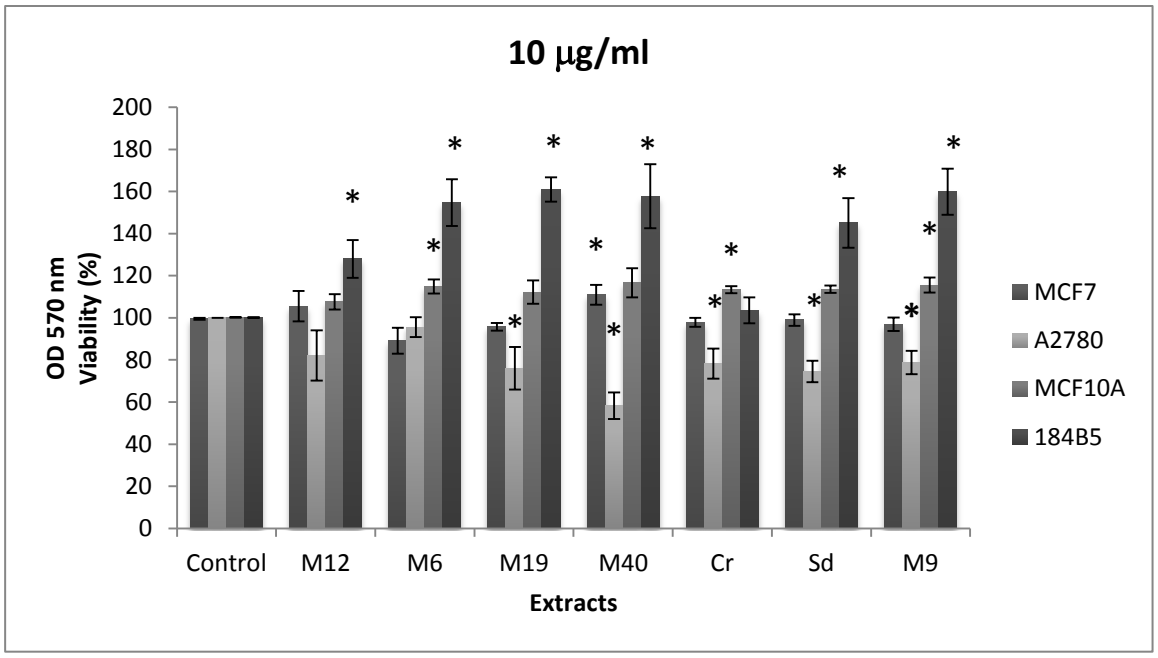

Figure 5.3C. Effect of 10 μg/ml of microalgal extracts on mammalian cell viability. All mammalian cells were exposed to 10 μg/ml of microalgal extracts and incubated for 24 hours. The cell viability was assessed by MTT assay ($^{*}P < 0.05$, T-test).

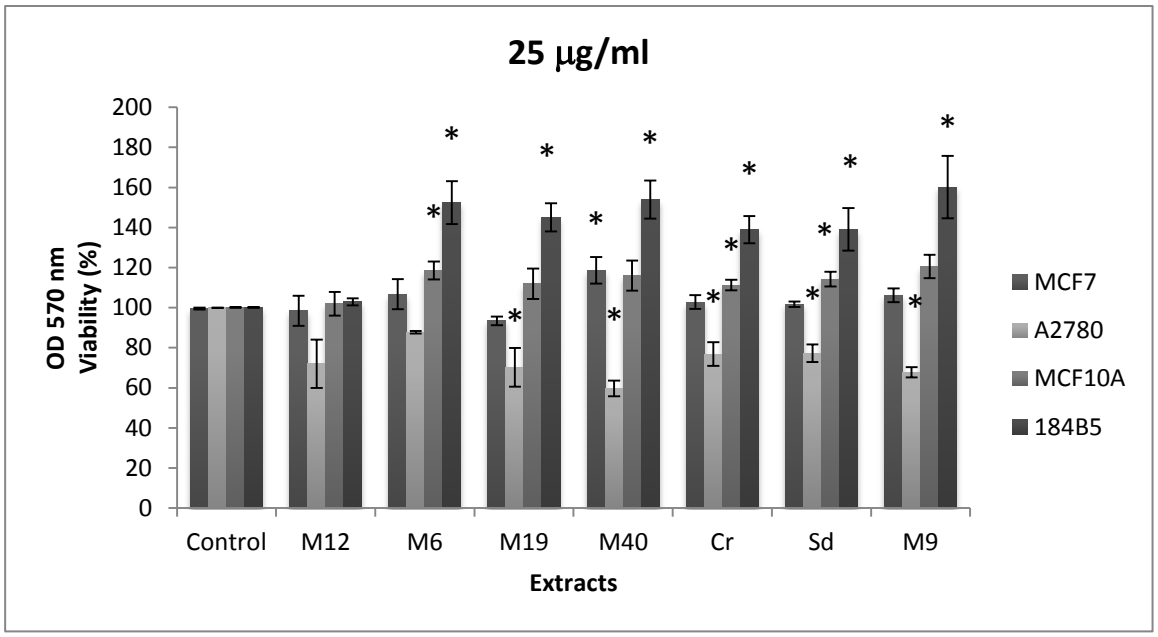

Figure 5.3D. Effect of 25 μg/ml of microalgal extracts on mammalian cell viability. All mammalian cells were exposed to 25 μg/ml of microalgal extracts and incubated for 24 hours. The cell viability was assessed by MTT assay ($^{*}P < 0.05$, T-test).

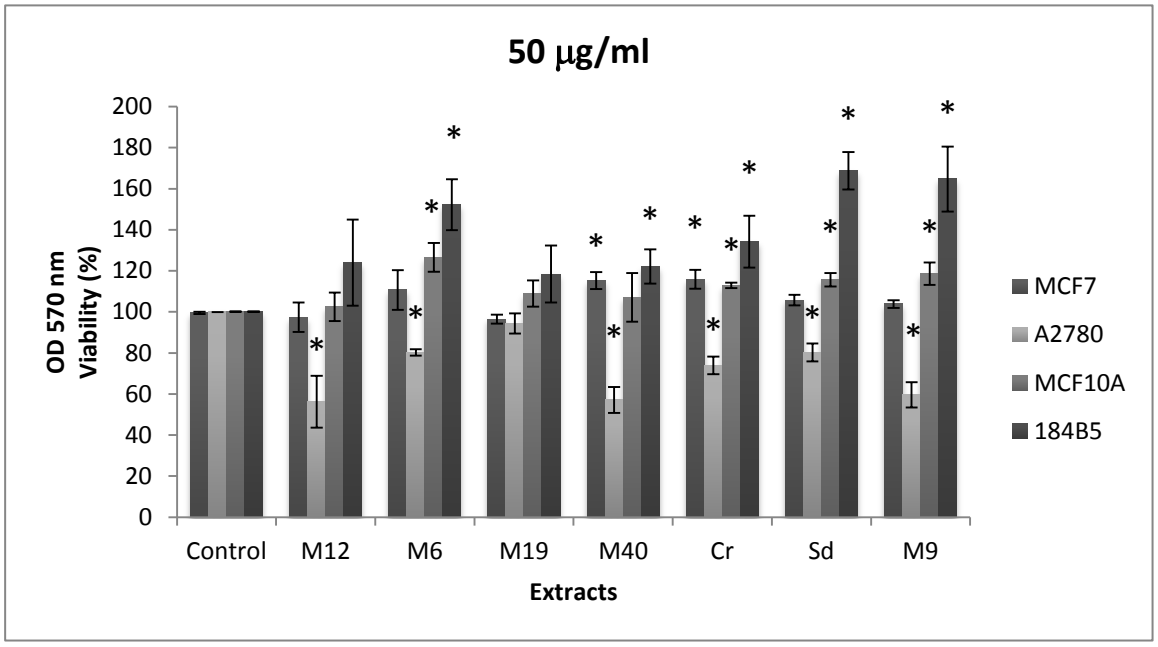

Figure 5.3E. Effect of 50 μg/ml of microalgal extracts on mammalian cell viability. All mammalian cells were exposed to 50 μg/ml of microalgal extracts and incubated for 24 hours. The cell viability was assessed by MTT assay ($^{*}P < 0.05$, T-test).

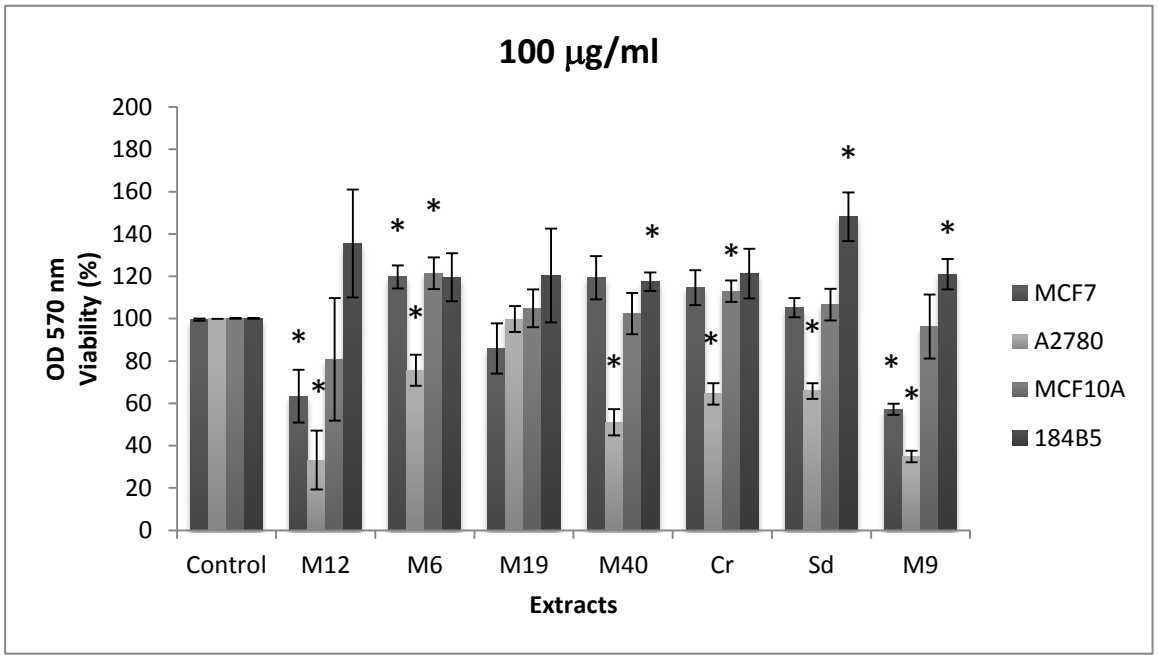

Figure 5.3F. Effect of 100 μg/ml of microalgal extracts on mammalian cell viability. All mammalian cells were exposed to 100 μg/ml of microalgal extracts and incubated for 24 hours. The cell viability was assessed by MTT assay ($^{*}P < 0.05$, T-test).

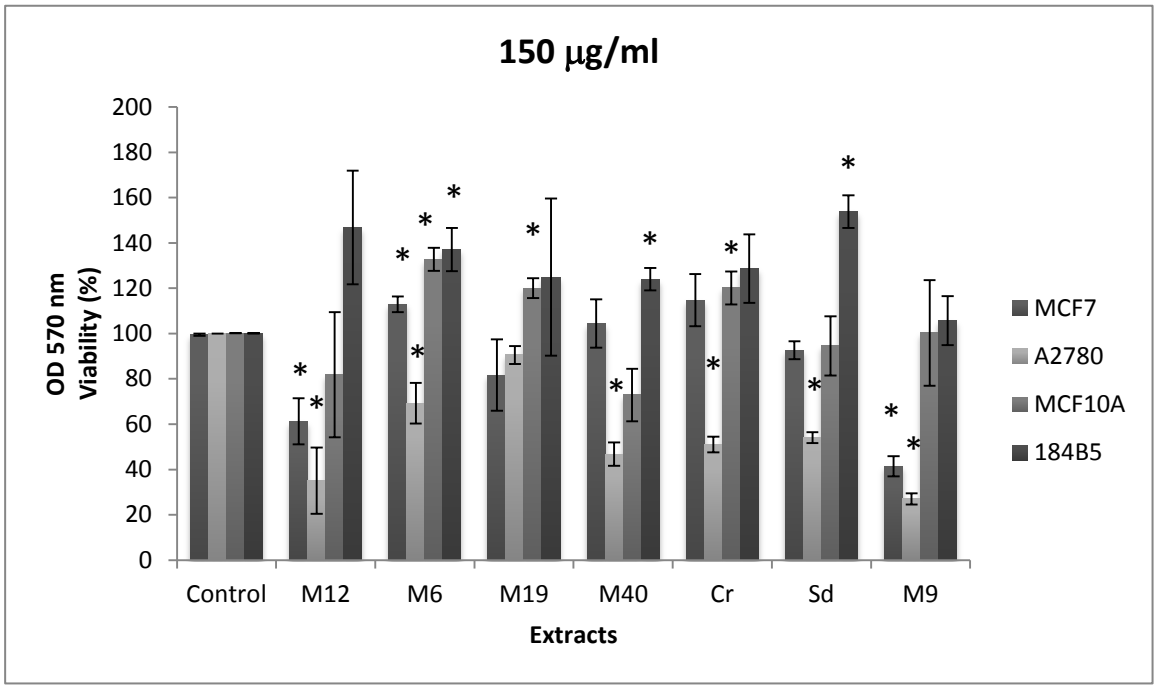

Figure 5.3G. Effect of 150 μg/ml of microalgal extracts on mammalian cell viability**.** All mammalian cells were exposed to 150 μg/ml of microalgal extracts and incubated for 24 hours. MTT assay was performed ($^{*}P < 0.05$, T-test).

DISCUSSION

Microalgal extracts have been associated with biological activities, including antibacterial (Senhorinho *et al.* 2015; Pina-Perez *et al.* 2017). From previous work on microalgal extract screening, however, extracts are obtained from biomass harvested in either the exponential or stationary growth phase (Cooper *et al.* 1983; Ordog *et al.* 2004), which seems to encompass the times for production of antibacterial compounds (Leflaive & Ten-Hage 2007). Debro and Ward (1979) tested one harvesting day during the exponential growth phase and one at stationary phase, and observed that most green microalgae had higher antibacterial activity at exponential phase rather than at stationary phase.

Antibacterial activity of freshwater green microalgae has not been previously systematically assessed over the growth curve and it is, therefore, not clear as to when is the earliest time to harvest microalgae when performing antibacterial activity screening. In order to determine if harvesting time plays an important role when screening for antibacterial activity, we harvested cells every seven days from early exponential to early stationary growth and tested the extracts against *S. aureus*.

Our results demonstrated that all wild *Chlamydomonas* sp. extracts showed similar patterns of antibacterial activity, with significantly higher activity detected in the exponential phase (day 35) compared to when cultures entered the stationary phase (day 49, Table 1). This represents an important finding, since to expedite the screening process it is key to know the earliest harvesting time.

The decrease of antibacterial activity at early stationary growth phase could indicate that the *Chlamydomonas* sp. produce less antibacterial compounds in this phase but also could suggest that these organisms start releasing the antibacterial compounds into the media. In this study, only intracellular extracts were evaluated. Noaman *et al*. (2004) observed the highest antibacterial activity from extracellular extracts of the cyanobacterium *Synechococcus leopoliensis* when cells were harvested during stationary phase of growth, indicating that the antibacterial compounds were released into the medium.

The microalgal strains from culture collections showed different results from those of the wild microalgae. Extracts from *C. reinhardtii* CPCC11 only showed antibacterial activity until day 28 (exponential phase). Moreover, as indicated by the MIC results (Table 1), extracts from this strain showed weaker antibacterial activity against *S. aureus* than any of the extracts from the wild *Chlamydomonas* sp. tested. Similarly, extracts from *S. dimorphus UTEX* 1237 only showed antibacterial activity at the beginning of the exponential phase, which is until day 14. This observation is supported by our earlier work in which no antibacterial activity from *S. dimorphus* was detected when screened at day 28 (Senhorinho *et al.* 2018). Debro & Ward (1979) observed that extracts from a strain of *C. reinhardtii* showed the same antibacterial activity at the exponential and stationary phases, which was not seen in our study. However, studies have shown that antibacterial activity of green microalgae may vary, not only among species, but also among strains, which may explain these discrepancies (Ordog *et al.* 2004; Aremu *et al.* 2014; Senhorinho *et al.* 2015).

Extracts M6 and M40 did not show any antibacterial activity on day 21, but was again detected on day 28. As microalgal metabolites are produced in very small amounts (Hernandez-

Carlos & Gamboa-Angulo 2011), a decrease in antibacterial compounds that occur during the growth cycle could potentially lead to negative results in the assay.

When extracts from a natural source demonstrate promising antibacterial activity, it is important to determine the effects on human cells. Even though extracts from a few microalgal species, including prokaryotic and eukaryotic, have been shown to decrease non-malignant and malignant cell viability (Bechelli *et al.* 2011), little is known about the effect of freshwater green microalgal extracts with antibacterial activity. As potential antibiotics, the compounds need to show potency against pathogenic organisms but low toxicity against mammalian cells (Olaizola 2003).

Therefore, in order to determine the effects of the microalgal extracts exhibiting antibacterial activity on mammalian cell viability, a viability test based on the reduction of 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) to a purple colour was performed. The microalgal extracts subsequently tested that showed promising activity against *S. aureus* resulted in no significant decrease in the observed cell viability of non-malignant mammalian cells, MCF10 and 184B5. This was observed even at the maximum extract concentration of 150 μg/ml (Figure 5.3.7). However, extracts analyzed from the wild microalgae inhibited *S. aureus* growth at 16 μg/ml to 128 μg/ml (Table 1), which were much lower levels than obtained with a culture collection strain, suggesting these extracts are potent and worthy of further investigation.

All the microalgal extracts tested decreased the viability of the malignant A2780 cell line at some concentration, with wild strains extracts M9, M12 and M40 exhibiting the most pronounced effects. Interestingly, extract M12 only had a negative effect on the cells at concentrations above

50 μg/ml, whilst extracts M9 and M40 started affecting the cells at lower concentrations (Figs. 3B, 3C). These results suggest that cytotoxicity, as is antibacterial activity, is extract dependent.

Extracts from the culture collection strains, Sd and Cr, significantly decreased cell viability of the A2780 line at all concentrations tested. However, these extracts did not decrease cell viability of any other cell line. This suggests that the active compounds present in these extracts exhibit selectivity over the ovarian cancer cells, which are rapidly dividing cells. Moreover, since extracts from the culture collection strains, Sd and Cr, did not exhibit promising antibacterial activity, it is possible that antibacterial activity and antitumor activity are not directly related.

The viability of MCF7 cells was significantly decreased by extracts M9 and M12, but only at the highest concentrations tested (Figures. 5.3.6, 5.3.7) and, interestingly, not by any other microalgal extract. Our results differ from the ones obtained by Ordog *et al*. (2004) who observed a decrease in MCF7 cell viability in cultures treated with all green microalgal extracts exhibiting antibacterial activity. However, different microalgal genera were used in our study, as well as specific concentrations of extracts, which may explain the discrepancies found with this study.

The wild microalgae tested in this study are originally from water bodies near abandoned mine sites, where high metal concentrations and low pH are commonly observed. Extract M9 was obtained from a *Chlamydomonas* sp. present in acidic water (pH 5.7) with high levels of iron while extract M12 came from a *Chlamydomonas* sp collected from an area with high levels of iron, nickel, copper and zinc (Senhorinho *et al.* 2018). Environmental stressors are known to contribute to microalgal adaptation (Leflaive & Ten-Hage 2007), which may lead these organisms to produce compounds that act on malignant cells.

Interestingly, it was observed that microalgal extracts at most concentrations significantly increased the viability of non-malignant 184B5 cells, and certain concentrations of extracts from M6, Cr, M9, M40 and Sd also increased the viability of non-malignant MCF10A cells. In particular, extracts from M6 and Cr increased the viability of MCF10A cells at all the concentrations tested, except for Cr at 5 µg/ml. The MTT assay used to test viability is a quantitative colorimetric assay, where the tetrazolium salt is reduced to a water-insoluble formazan mainly by the succinate dehydrogenase enzyme of active mitochondria (Chakrabarti *et al.* 2000). Toxic compounds that damage the mitochondria of cells and reduce their viability, decrease the reduction of tetrazolium salt to formazan (Mueller 2004). Therefore, the absorption of the dissolved formazan usually correlates to the number of viable cells with active mitochondria or metabolism (Riss *et al*, 2016). The high MTT readings observed with the 184B5 and MCF10A cells, would suggest that certain compounds found in microalgal extracts increase cell viability/proliferation of the non-malignant cells. However, it is also possible for some compounds to increase the mitochondrial enzymatic activity, thereby increasing MTT absorbance, without having an effect on cell number or cell viability (Pagliacci *et al.* 1993, Chakrabarti *et al.* 2000, Wang *et al.* 2010). Since a large number of compounds are likely present in the methanolic extracts of green microalgae (Annamalai & Nallamuthu 2014), it is possible that some of them increased mitochondrial metabolism, particularly of the 184B5 line. Therefore, further studies should look to identify and isolate the antibacterial compounds and their effects on human cells should be evaluated.

MCF7 is a breast carcinoma cell line with slowly proliferating cells that express high levels of cytoplasmic estrogen receptors (Lee *et al.* 2015), whilst A2780 is an ovarian cancer cell line from an endometrioid adenocarcinoma tumor that rapidly proliferates. A2780 cells were

significantly more affected by the microalgal extracts than MCF7 cells (Fig 3). This suggests the presence of cytotoxic compounds in the microalgal extracts. Cytotoxic drug compounds work by preferentially killing rapidly proliferating tumor cells (Mitchison 2011) whilst slowly proliferating cells are less sensitive (Strese *et al.* 2013), which was seen in this study.

Some variation was observed in the cell viability results when the replicates of the microalgal extracts were tested; particularly extract M12 at high concentrations (Figs. 3F, 3G). Microalgal extracts, as with other naturally sourced mixed biocompounds, have been shown to exhibit some variation in the concentrations produced during cultivation, and may even stop production of desired compounds (Ploutno & Carmeli 2000; Wright 2014), which is likely to explain the variation observed in this study.

Overall, the results of the present study demonstrated, therefore, that harvesting time is very important when evaluating antibacterial activity of freshwater green microalgae. Intracellular extracts from wild strains of *Chlamydomonas* sp. exhibited higher antibacterial activity against *S. aureus* in the exponential growth phase, and have lower activity at stationary phase. Moreover, different microalgal genera and different strains demonstrate different patterns of antibacterial activity production during cell growth, as observed with the wild and the laboratory strain microalgae.

Furthermore, methanolic extracts from freshwater green microalgae at the concentrations tested have the ability to decrease viability of malignant cells, particularly the rapidly proliferating cell line A2780, but not non-malignant cells MCF10A and 184B5. Therefore, these results suggest that extracts of freshwater green microalgae are worthy of further investigation as antibiotics that are not cytotoxic to human cells. Additionally, since the results indicate that green

microalgal extracts could harbour compounds that preferentially target malignant cells, more research is warranted needed to directly evaluate the compounds involved in this activity.

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CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

The lack of new antibiotics represents a major concern for the progress of the modern medicine and the ability to directly save the lives of patients. The worldwide increase in bacterial resistance has created urgency for the discovery of novel antibiotic compounds and new bacterial targets to allow continuation of successful treatment of bacterial infections. Since most available antibiotics are restricted to actinomycetes and fungi from soil, which have have not provided any new compounds over the past decades (Wright 2012), alternative microbial sources could represent important targets for new antibiotics.

The premise of this work was for the first time to analyze the potential of freshwater green microalgae found in water bodies near abandoned mine sites as antibiotic producers. Chemical analysis of these waters revealed that most of them were contaminated with metals and/or had a low pH (Table S1). The results of screening demonstrated that methanolic extracts obtained from certain microalgae isolated from these environments did exhibit antibacterial activity against Gram-positive bacteria, particularly *S. aureus*. Furthermore, compared to microalgae screened previously (Bhagavathy *et al*. 2011; Challouf *et al.* 2012), their extracts also exhibited better potential as antibacterial producers in terms of significantly lower minimum inhibitory concentrations (MICs).

The antibacterial activity detected in green microalgal extracts was expected to correlate with the concentrations of heavy metals analysed and/or low pH detected by the water analysis. However, there was no statistical evidence that green microalgae, exhibiting promising antibacterial activity, have a direct association with environments with high concentrations of the metals analyzed or low pH. Nonetheless, based on the high occurrence rate of green microalgae

showing antibacterial activity, as well as low MIC values, there would appear that an association between these extreme environments and the antibacterial activity of microalgae is still likely to exist.

Therefore, future studies should consider systematically investigating freshwater near abandoned mine sites for factors associated with the antibacterial activity. Factors such as ecological interaction (including with bacteria), which could be evaluated by molecular technologies such as polymerase chain reaction and/or DNA sequencing, could lead to a positive microbial association with antibacterial producer microalgae. Furthermore, the impact of very high seasonal temperature variations and extended periods of ice cover in Northern Ontario water bodies was not considered in this study. This could be associated with antibacterial activity of green microalgae, since a temperature correlation has been previously observed with macroalgae exhibiting antibacterial activity (Amade & Lemee 1998). Thus, studies on the effect of temperature variation on the antibacterial activity of microalgae could be an important avenue to be explored.

An important part of this study was the evaluation of timing of biomass harvesting and antibacterial activity of green microalgal extracts. Microalgae from extreme environments have not been previously analysed during the growth curve for antibacterial activity. The results demonstrated that wild *Chlamydomomonas* sp. analyzed did indeed exhibit variation in antibacterial production during cell growth, with the maximum output towards the end of the exponential phase. This highlights that screening biomass harvested for antibacterial activity should not be performed at a random time during growth, as concentrations of active compounds may be too low to be detected. Moreover, this information is essential for further studies on identification and isolation of antibacterial compounds, as the cells should be harvested during

the highest peak of antibacterial activity, as the compounds are likely to be at higher concentrations. This finding is, therefore, important for further mass screening studies.

In this study we also determined that microalgal extracts showing antibacterial activity did not decrease the cell viability of evaluated non-malignant cell lines. This result further suggests that these microorganisms are worthy of further investigation for antibiotic production, and that determination of the specific active antibacterial compound(s) should be pursued. Analysis of metabolites in the microalgal extracts, showing promising antibacterial activity, suggested that they are a rich source of amino acids, which are usually associated with the production of secondary metabolites (Demain 1998). Future experimental programs should include fractionation of the most promising extracts following by identification and isolation of the active compound(s).

Furthermore, a very interesting finding of this study was the decreased cell viability of human malignant cells treated by microalgal extracts, particularly with the rapidly dividing ovarian carcinoma A2780 line. This cell line also seemed to be selectively targeted over the slowly dividing malignant cells, MCF7. Consequently, future studies should include evaluation of the effects of the microalgal extracts on rapidly dividing malignant cells.

There is undoubtedly a great worldwide need for new molecules with antibacterial activity. Antimicrobial resistance kills 700,000 people per year and it has been estimated that these numbers will increase to 10 million deaths per year by 2050, if the trend does not change (O'Neill 2014). The results of the present study offer a new insight into the importance of unexplored microalgae from extreme environments as antibiotic producers. Although very promising results were obtained, significant further study is necessary before antibacterial

molecules from microalgae can undergo clinical investigation. The determination of the active molecules should, therefore, be the next step towards evaluating these extracts. This should then be followed by assessing the compounds with antibacterial resistant microorganisms, such as methicillin resistant *S. aureus*, followed by toxicity and bioavailability of the active compounds.

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APPENDICES

Table S1: Chemical analysis of water samples harbouring microalgae exhibiting (positive) and not exhibiting (negative) antibacterial activity.

	Water chemistry							
	Fe	Ni	Cu	Zn	As	Cd	Pb	pH
Standard[1]	0.3	0.025	0.002	0.03	0.005	0.00009	0.001	6.5-9
Positive samples								
M23	21.5	0.673	0.803	2.53	0.002	0.001	0.008	2.9
M2	8.680	0.144	0.040	42.40	0.002	0.675	0.050	2.9
M9	1.317	<0.001	<0.001	0.006	0.0005	<0.001	<0.001	5.7
M12	0.670	0.208	0.066	0.067	<0.001	<0.001	<0.001	6.9
M18	0.363	0.001	0.001	0.003	<0.001	<0.001	<0.001	6.5
M4	0.323	<0.001	0.002	0.010	0.001	<0.001	<0.001	6.0
M40	0.231	0.002	0.006	<0.001	0.002	<0.001	<0.001	7.2
M15	0.088	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	7.6
M19	0.058	0.001	<0.001	0.001	<0.001	<0.001	<0.001	7.2
M17	0.050	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	7.1
M6	0.045	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	8.0
M13	0.043	0.001	<0.001	0.001	<0.001	<0.001	<0.001	6.9
M16	0.040	<0.001	0.001	0.002	<0.001	<0.001	<0.001	7.0
M21	0.029	<0.001	<0.001	0.003	<0.001	<0.001	<0.001	6.2
M20	0.023	<0.001	0.001	0.001	<0.001	<0.001	<0.001	6.8

Table S1. Continued.

Water chemistry								
Standard[1]	Fe 0.3	Ni 0.025	Cu 0.002	Zn 0.03	As 0.005	Cd 0.00009	Pb 0.001	pH 6.5-9
Negative samples								
M38	4.58	0.257	0.671	16.9	0.002	0.070	1.76	4.4
M8	1.014	0.001	0.001	0.005	0.0005	<0.001	<0.001	5.8
M37	0.884	0.025	0.011	0.035	<0.001	<0.001	<0.001	7.4
M36	0.612	0.016	0.020	0.012	0.008	<0.001	0.003	7.8
M11	0.478	<0.001	<0.001	0.001	0.0004	<0.001	<0.001	6.6
M10	0.349	<0.001	<0.001	0.002	0.0006	<0.001	<0.001	6.5
M35	0.338	0.002	0.117	0.034	0.002	<0.001	<0.001	6.7
M27	0.323	0.018	0.002	0.001	<0.001	<0.001	<0.001	7.6
M14	0.289	0.005	0.001	0.001	<0.001	<0.001	<0.001	7.4
M31	0.207	0.002	0.002	0.001	<0.001	<0.001	<0.001	5.7
M5	0.166	<0.001	<0.001	0.005	<0.001	<0.001	<0.001	4.5
M26	0.164	0.003	0.001	0.006	<0.001	<0.001	<0.001	5.3
M24	0.155	0.006	0.012	0.009	0.002	0.018	<0.001	7.9
M28	0.151	0.004	0.003	<0.001	<0.001	<0.001	<0.001	8.4
M1	0.136	<0.001	<0.001	0.002	<0.001	<0.001	0.001	7.4

Table S1. Continued.

Water chemistry								
	Fe	Ni	Cu	Zn	As	Cd	Pb	pH
Standard[1]	0.3	0.025	0.002	0.03	0.005	0.00009	0.001	6.5-9
Negative samples								
M34	0.130	0.014	0.084	0.030	0.002	<0.001	<0.001	7.0
M3	0.130	0.003	0.003	0.003	0.003	<0.001	<0.001	8.1
M25	0.107	0.007	0.004	0.003	0.007	<0.001	<0.001	8.0
M7	0.090	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	7.9
M33	0.081	0.002	0.137	<0.001	<0.001	<0.001	<0.001	7.4
M30	0.066	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	5.8
M29	0.042	0.001	0.001	<0.001	<0.001	<0.001	<0.001	6.7
M32	0.039	0.001	0.282	<0.001	<0.001	<0.001	<0.001	6.0
M39	0.050	0.003	0.004	0.001	<0.001	<0.001	<0.001	6.9
M22	0.001	0.010	0.042	0.007	0.200	<0.001	0.001	8.0

[1]Standard (ppm) is based on the Canadian water quality guidelines for protection of aquatic life (CCME, 2017).

Table S2. Metabolites in promising microalgal extracts exhibiting antibacterial activity.

Metabolite	Microalgal Extracts						
Amino acids (μM)	M6	M9	M12	M15	M19	M20	M40
Alanine	23.9	30.2	145	1060	1520	809	3
Arginine	0.939	0.635	3.35	111	212	1.89	31.2
Asparagine	45.2	12.3	5.03	525	682	16.2	12.5
Aspartic acid	0.453	0.683	0.432	97.2	85.9	0.654	0.564
Citruline	1.67	1.4	2.63	12.7	13.4	5.19	1.5
Glutamine	3.32	2.77	23.4	67.8	131	9.34	11.7
Glutamic acid	42.5	52.5	106	1210	1740	811	346
Glycine	4.73	10.1	18.5	97.2	147	22.1	15.2
Histidine	1.21	0.483	0.291	2.63	2.94	5.49	0.663
Isoleucine	3.86	4.7	10.5	17.6	24.1	24.4	8.43
Leucine	10.3	9.29	20.8	33.7	48.3	35.1	19.7
Methionine	1.49	1.5	1.45	1.86	1.46	1.69	2.02
Phenylalanine	2.15	2.63	5.25	15.3	21.3	7.1	4.36
Proline	4.36	0.339	6.97	21.9	34.9	105	4.03
Serine	7.47	16.6	31.9	473	751	53.1	48.1
Threonine	5.59	7.86	16.3	47.7	65.1	31.6	22.2
Tryptophan	0.149	0.411	1.08	6.75	8.79	5.66	0.855
Tyrosine	0.974	1.34	6.92	14.4	22.2	7.92	2.66
Valine	7.3	8.54	21.8	42	56.8	52.5	22.9

Table S2. Continued.

Metabolite	Microalgal Extracts						
Glycerophospholipids (μM)	M6	M9	M12	M15	M19	M20	M40
LysoPhosphatidylcholine acyl	11.7	15.1	47.4	4.92	17.3	0.124	0.129
LysoPhosphatidylcholine acyl	16.4	6.04	5.36	7.07	24.9	5.1	3.87
Phosphatidylcholine diacyl C32:3	1.1	2.65	14.5	1.39	5.97	0.028	0.013
Phosphatidylcholine diacyl C34:1	4.15	0.876	5.18	1.08	6.32	1.02	0.073
Phosphatidylcholine diacyl C34:2	18.5	6.35	29	4.48	20	2.47	0.145
Phosphatidylcholine diacyl C34:3	34.1	35	149	6.56	27.9	0.193	0.102
Phosphatidylcholine diacyl C34:4	5.6	9.93	39.5	1.39	6.34	0.046	0.032
Phosphatidylcholine diacyl C36:3	3.72	1.12	8.3	2.63	12.5	0.225	0.041
Phosphatidylcholine diacyl C36:4	12.3	3.41	23.7	4.49	20.9	0.149	0.055
Phosphatidylcholine diacyl C36:5	28.3	7.8	33.4	4.96	20.2	0.104	0.083
Phosphatidylcholine diacyl C36:6	56.6	30.3	110	3.37	13.8	0.065	0.087
Phosphatidylcholine diacyl C36:0	7.94	8.99	31.7	0.424	1.86	0.129	0.193
Sphingolipids (μM)							
Hydroxysphingomyeline C22:2	0.043	0.022	0.121	0.136	0.514	0.02	0.014
Hydroxysphingomyeline C16:1	0.039	0.015	0.052	0.012	0.046	0.019	0.024
Sphingomyeline C20:2	0.069	0.032	0.247	0.041	0.014	0.006	0.008
Sphingomyeline C24:0	0.324	0.15	0.172	0.178	0.203	0.606	0.137

Table S2. Continued.

Metabolite	Microalgal Extracts						
Acylcarnitines (μM)	M6	M9	M12	M15	M19	M20	M40
Decanoylcarnitine	0.17	0.146	0.044	0.072	0.076	0.078	0.199
Decenoylcarnitine	0.238	0.277	0.195	0.261	0.226	0.293	0.312
Biogenic amines (μM)							
Alpha-aminoadipic acid	0.145	0.432	0.543	12.8	10.6	0.701	1.21
Methioninesulfoxide	1.38	0.764	5.76	9.48	6.11	6.16	2.74
cis-OH-pro	1.29	1.29	1.29	1.46	1.38	1.29	1.29
Putrescine	0.364	0.969	0.976	0.0825	0.0145	0.123	0.0899
Taurine	1.2	0.452	0.321	3.99	5.56	0.517	0.452